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Crosstalk between ceramide and cell signalling pathways controlling chronological lifespan in yeast

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Ó tocadora de harpa, se eu beijasse

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Teu gesto, sem beijar as tuas mãos!
E, beijando-o, descesse pelos desvãos
Do sonho, até que enfim eu o encontrasse.

Tornado Puro Gesto, gesto-face
Da medalha sinistra — reis cristãos
Ajoelhando, inimigos e irmãos,
Quando processional o andor passasse!

Teu gesto que arrepanha e se extasia.
O teu gesto completo, lua fria
Subindo, e em baixo, negros, os juncais.

Caverna em estalactites o teu gesto.
Não poder eu prendê-lo, fazer mais
Que vê-lo e que perdê-lo! E o sonho é o resto.

Fernando Pessoa

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AbA, Aureobasidin A; **ADP**, Adenosine diphosphate; **ALP**, Alkaline phosphatase; **AMPK**, 5' Adenosine monophosphate- activated protein kinase; **AP-1**, Activator protein 1 transcription factor; **ATG**, Autophagy-related gene; **ATP**, Adenosine triphosphate; **CAPP**, Ceramide-activated protein Phosphatase; **CK2**, Casein kinase 2; **CL**, Cardiolipin; **CLS**, Chronological lifespan; **CMA**, Chaperone-mediated autophagy; **COX**, Cytochrome *c* oxidase; **CPY**, Carboxypeptidase Y; **CR**, Calorie restriction; **Cvt**, Cytoplasm-to-vacuole targeting; **CWI**, Cell Wall Integrity; **DAG**, Diacylglycerol; **DHE**, Dihydroethidium; **DHS**, Dihydrosphingosine; **DMSO**, Dimethyl sulfoxide; **DNA**, deoxyribonucleic acid; **DNP**, 2-4 dinitrophenol; **DTT**, Dithiothreitol; **EDTA**, Ethylenediamine tetraacetic Acid; **ER**, Endoplasmic reticulum; **ERAD**, Endoplasmic-Reticulum-associated protein degradation; **ERMES**, Endoplasmic Reticulum (ER)-mitochondria encounter structure; **ETC**, Electron transport chain; **FACS**, Fluorescence-activated cell sorting; **FCCP**, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; **Fw**, forward; **GFP**, Green fluorescent protein; **GTP**, Guanosine triphosphate; **GSH**, Glutathione; **HM**, hydrophobic motif; **HA**, Human influenza hemagglutinin; **HOG**, High Osmolarity Glycerol; **HSC**, Hematopoietic stem cells; **IGF-1**, Insulin-like growth factor-1; **IL-13**, Interleukin-13; **IPC**, Inositol-phosphorylceramide; **ISC1**, Inositol phosphosphingolipid phospholipase C; **JNK**, c-Jun N-terminal protein Kinase; **LAMP-2A**, Lysosomal associated membrane protein-2A; **LCBs**, Long chain bases; **LCBPs**, Phosphorylated long chain bases; **LC-MS/MS**, Liquid chromatography-tandem mass spectrometry; **MAPK**, Mitogen-activated protein kinases; **MIPC**, Mannosyl-inositol-phosphorylceramide; **M(IP)$_2$C**, Mannosyl-diinositol-phosphorylceramide; **MNF 1,2**, Mitofusins 1,2; **MOPS**, 3-(N-morpholino)propanesulfonic Acid; **mtUPR**, mitochondrial unfolded protein response; **NADH**, Nicotinamide adenine dinucleotide; **NADPH**, Nicotinamide adenine dinucleotide phosphate; **NCR**, Nitrogen catabolite repression; **NTCB**, 2-nitro-5-thiocyanatobenzoic acid; **PAS**, Phagophore Assembly Site; **PBS**, Phosphate buffer saline; **PCR**, Polymerase Chain Reaction; **PDS**, Post-Diauxic Shift; **PDK1**, 3-phosphoinositide dependent protein kinase-1; **PG**, Phosphatidylglycerol; **PH**, Pleckstrin homology; **PHS**, Phytosphingosine; **PI**, Propidium iodide; **PI3K**, Phosphoinositide 3-Kinase; **PIPES**, Piperazine-N,N-bis(2-

ethanesulfonic acid); **PKA**, Protein Kinase A; **PKB/Akt**, Protein Kinase B; **PKC**, Protein Kinase C; **PMSF**, Phenylmethanesulfonyl Fluoride; ***p*-NPP**, para-Nitrophenylphosphate; **PP1**, Protein Phosphatase type 1; **PP2A**, Protein Phosphatase type 2A; **PS**, Phosphatidylserine; **Rb**, Retinoblastoma; **RLS**, Replicative lifespan; **RNA**, Ribonucleic acid; **ROS**, Reactive oxygen species; **Rv**, reverse; **S1P**, Sphingosine-1-phosphate; **SAHF**, Senescence-associated heterochromatin foci; **SAP**, SIT4-associated proteins; **SC**, Synthetic complete; **SD**, Standard deviation; **SDS-PAGE**, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **SNARE**, Soluble NSF attachment receptor; **SPH**, Sphingosine; **SPT**, Serine palmitoyltransferase; **TCA**, Trichloroacetic acid; **TM**, turn motif; **TORC1**, Target Of Rapamycin Complex 1; **TORC2**, Target Of Rapamycin Complex 2; **UPR**, Unfolded Protein Response; **UPRE**, Unfolded Protein Response Element; **UV**, Ultraviolet; **vps**, Vacuolar protein sorting; **YPD**-Yeast Extract Peptone Dextrose; **YPL**-Yeast Extract Peptone Lactate.

Summary

Sphingolipids regulate crucial biological processes, such as stress response and apoptosis. The budding yeast *Saccharomyces cerevisiae* has been pivotal to uncover the impact of sphingolipid dynamics on eukaryotic cell physiology and metabolism. The bioactive sphingolipid ceramide can be generated by the *de novo* biosynthetic pathway or through the hydrolysis of complex sphingolipids, the last catalysed by the inositol phosphosphingolipid phospholipase C protein, Isc1p (the yeast orthologue of the mammalian neutral sphingomyelinase 2). Cells lacking Isc1p display shortened chronological lifespan (CLS), oxidative stress sensitivity and severe mitochondrial dysfunctions, which are associated with iron overload, enhanced oxidative damage to biomolecules and increased cell death by Yca1p-mediated apoptosis. Previous results demonstrated that the PP2A-like phosphatase Sit4p and the MAPK kinase Hog1p are activated in response to increased ceramide levels displayed by *isc1*Δ cells during aging and contribute to mitochondrial dysfunction, hydrogen peroxide hypersensitivity and shortened chronological lifespan of *isc1*Δ cells.

In this thesis, the role of the nutrient-sensing Target Of Rapamycin Complex 1 (TORC1) and its downstream effector, the AGC protein kinase Sch9p, in *isc1*Δ phenotypes was assessed. This work demonstrates that the TORC1-Sch9p pathway is activated in cells lacking Isc1p. The deletion of *TOR1* or *SCH9* abolishes the premature aging, oxidative stress sensitivity and mitochondrial dysfunctions displayed by *isc1*Δ cells and this is correlated with alterations in autophagic activity and flux. The protective effect of *TOR1* deletion is not associated with the attenuation of Hog1p hyperphosphorylation, which was previously implicated in *isc1*Δ phenotypes. Importantly, Hog1p activation is responsive to ceramide by Sch9p-dependent mechanisms. Our data support a model in which Isc1p regulates mitochondrial function and chronological lifespan in yeast through the TORC1-Sch9p pathway, although Isc1p and TORC1 also seem to act through independent pathways, as *isc1*Δ*tor1*Δ phenotypes are intermediate to those displayed by *isc1*Δ and *tor1*Δ cells. We then provide evidence the TORC1-Sch9p signalling pathway acts as a central axis to integrate upstream Isc1p-driven ceramide signalling signals to downstream effectors Sch9p and Sit4p.

Macroautophagy plays an important regulatory role in mitochondrial function, stress response and aging. Since these processes are impaired in Isc1p-deficient cells, we aimed to investigate how Isc1p-driven ceramide signalling regulates macroautophagy and other mitochondrial quality control mechanisms, namely mitochondrial dynamics and the selective degradation of mitochondria by mitophagy. The results show that Isc1p deficiency reduces autophagic flux by primarily impairing vesicular trafficking, vacuolar proteolysis, acidification and morphology. These phenotypes are suppressed by downregulation of TORC1 and its downstream effectors, Sch9p and Sit4p, which integrate nutrient and stress signals from TORC1 with ceramide signalling from Isc1p. We show that the deletion of *ISC1* leads to hyperactivation of mitophagy, presumably as an adaptive response to mitochondrial dysfunction, and this was correlated with loss of cell viability of *isc1*Δ cells. Isc1p-deficient cells also displayed higher levels of the mitochondrial fission protein Dnm1p associated with unbalanced mitochondrial fission, contributing to mitochondrial fragmentation, oxidative stress sensitivity and shortened lifespan. The deletion of *TOR1*, *SCH9*, *SIT4* and *HOG1* attenuated the induction of mitophagy in respiratory conditions, and this was correlated with the suppression of mitochondrial fragmentation and improvement of mitochondrial function. We further show that Isc1p and Dnm1p physically interact *in vitro*, further supporting a possible regulatory role for Isc1p in mitochondrial dynamics.

The deletion of *SIT4* extends lifespan in yeast by boosting mitochondrial function (mitochondrial catabolic derepression) and stress response. Although Sit4p functions downstream of Isc1p, the phosphatase is downregulated by TORC1 and was recently shown to control the turnover of complex sphingolipids in a Npr1p-dependent manner. However, how TORC1 effector Sit4p integrates nutrient and sphingolipid signalling is not yet fully understood. Our results demonstrate that Sit4p regulates the sphingolipid biosynthetic pathway by a transcriptional regulatory mechanism involving the ceramide synthase *LAG1* and ceramidase *YPC1*. An increased LCBPs/LCBs ratio is observed in *sit4*Δ cells and ceramide metabolism is preferably shunted towards the synthesis of α-hydroxylated phytoceramide species, which have been implicated on proper mitochondrial function. Importantly, overall decreased ceramide production in *sit4*Δ cells is correlated with reduced activation of the TORC2-Pkh1p-Ypk1p pathway and decreased Orm phosphorylation. Moreover, the Pkh1/2p-Sch9p

pathway is downregulated in *sit4*Δ cells by a Snf1p-dependent mechanism. The downregulation of this pathway is associated with improved mitochondrial fitness and extended CLS upon Sit4p deficiency. Given the regulatory role of Sit4p in nutrient signalling and the regulation of the activation of the Pkh1/2p-Sch9p pathway, our results provide evidence that Sit4p is not only an effector but also a key regulator of sphingolipid metabolism and is involved in a regulatory network of interacting pathways that integrate signals from TORC1-mediated nutrient sensing with sphingolipid dynamics to regulate cell growth and longevity in yeast.

In conclusion, the work described in this thesis provides new insights on the molecular mechanisms by which cellular signalling pathways are activated in response to alterations in sphingolipid metabolism and the impact on crucial cellular functions and metabolism. This clarification is pivotal to understand sphingolipid functions in more detail and define new strategies to improve human health and extend lifespan.

Sumário

Os esfingolípidos regulam processos biológicos cruciais, tais como a resposta ao estresse e apoptose. A levedura *Saccharomyces cerevisiae* tem sido crucial para definir o impacto da dinâmica do metabolismo dos esfingolípidos na fisiologia e metabolismo celular dos eucariontes. O esfingolípido bioactivo ceramida pode ser gerado pela via biossintética *de novo* ou através da hidrólise dos esfingolípidos complexos, sendo que o último processo mencionado é catalisado pela proteína Isc1p (inositol phosphosphingolipid phospholipase C protein), que é ortóloga à proteína esfingomielinase neutra tipo 2 em mamíferos. As células deficientes na proteína Isc1p apresentam envelhecimento cronológico prematuro, sensibilidade aumentada ao estresse oxidativo e disfunção mitocondrial severa, os quais estão associados com o excessivo influxo de ferro, aumento dos danos oxidativos a biomoléculas e aumento de morte celular por apoptose mediado pela metacaspase Yca1p. Estudos anteriores demonstraram que a proteína fosfatase da família das proteínas fosfatases tipo 2A e a proteína da via das proteínas cínases activadas por mitogénios (MAPK) Hog1p são activadas em resposta ao aumento dos níveis de ceramida exibidos pelas células deficientes na proteína Isc1p durante o envelhecimento, contribuindo, desta forma, para a disfunção mitocondrial severa, sensibilidade aumentada ao estresse oxidativo e envelhecimento cronológico prematuro apresentadas por este mutante.

Nesta tese, o papel desempenhado pela via de sinalização mediada por nutrientes, a via Target Of Rapamycin Complex 1 (TORC1) e a sua proteína efectora, a proteína cínase Sch9p, nos fenótipos de células deficientes na proteína Isc1p foi analisado. Este trabalho demonstra que a via sinalizadora TORC1-Sch9p está mais activa no mutante *isc1*Δ. A deleção dos genes *TOR1* e *SCH9* suprime o envelhecimento cronológico prematuro, sensibilidade aumentada ao estresse oxidativo e disfunção mitocondrial severa ostentadas pelo mutante *isc1*Δ e tal está associado com a supressão do defeito na autofagia exibido pelo mutante. O efeito protector promovido pela deleção do gene *TOR1* não está relacionado com a activação da via sinalizadora mediada pela proteína Hog1p, a qual está associada aos fenótipos exibidos pelas células deficientes na proteína Isc1p. Por outro lado, a activação de Hog1p em resposta a ceramida ocorre por

mecanismos dependentes de Sch9p. Os dados apresentados neste trabalho suportam um modelo segundo o qual a proteína Isc1p regula a função mitocondrial e o envelhecimento cronológico em levedura através da via sinalizadora TORC1-Sch9p, embora existam também mecanismos independentes entre Isc1p e TORC1, uma vez que os fenótipos apresentados pelo mutante *isc1*Δ*tor1*Δ são intermédios àqueles exibidos pelas células mutantes *isc1*Δ e *tor1*Δ. Neste sentido, o trabalho oferece uma nova perspectiva segundo a qual a via sinalizadora TORC1-Sch9p actua como um eixo central que integra sinalização derivada da ceramida gerada pela proteína Isc1p e a transmite às proteínas efectoras Sch9p e Sit4p.

A macroautofagia desempenha um papel importante na regulação da função mitocondrial, resposta ao estresse e longevidade. Dado que estes processos estão desregulados nas células deficientes na proteína Isc1p, este trabalho pretende investigar de que forma a sinalização derivada da ceramida gerada por intermédio da proteína Isc1p regula a macroautofagia e outros mecanismos de controlo de qualidade mitocondrial, nomeadamente a dinâmica mitocondrial e a degradação selectiva de mitocôndrias pelo processo de mitofagia. Os resultados demonstram que a ausência de Isc1p reduz o fluxo autofágico ao comprometer primariamente o tráfego vesicular, a proteólise, acidificação e morfologia vacuolar. Estes fenótipos são suprimidos pela diminuição na actividade do complexo proteico TORC1 e das proteínas efectoras, Sch9p e Sit4p, as quais integram sinalização por nutrientes e estresse derivada de TORC1 com a sinalização por ceramida gerada por Isc1p. Por outro lado, foi demonstrado que a deleção do gene *ISC1* promove a hiperactivação da mitofagia, presumivelmente em resposta à disfunção mitocondrial, e tal está associado com a perda de viabilidade celular do mutante *isc1*Δ. As células deficientes na proteína Isc1p também exibem níveis aumentados da proteína mitocondrial de fissão Dnm1p e tal está relacionado com fissão mitocondrial excessiva, contribuindo, desta forma, para a fragmentação da rede mitocondrial, sensibilidade aumentada ao estresse oxidativo e envelhecimento cronológico prematuro. De forma notável, a deleção dos genes *TOR1*, *SCH9*, *SIT4* e *HOG1* atenuam a indução da mitofagia, em condições respiratórias, e tal está correlacionado com a supressão da fragmentação mitocondrial e melhoramento da função mitocondrial. Este trabalho também demonstra que as proteínas Isc1p e Dnm1p interagem

fisicamente *in vitro,* o que suporta um possível papel regulatório da proteína Isc1p no processo de dinâmica mitocondrial.

A deficiência do gene *SIT4* estende a longevidade em levedura ao melhorar a função mitocondrial (desrepressão catabólica da mitocôndria) e a resposta ao estresse. Embora a proteína Sit4p seja efectora da proteína Isc1p, a fosfatase é inibida pelo complexo TORC1 e recentemente foi demonstrado que a proteína controla o metabolismo dos esfingolípidos complexos por um processo dependente da proteína cínase Npr1p. Contudo, ainda é desconhecido de que forma a proteína Sit4p integra a sinalização mediada por nutrientes e esfingolípidos para regular a longevidade celular. Desta forma, este trabalho pretende estabelecer uma associação funcional entre Sit4p e o metabolismo dos esfingolípidos e avaliar o seu impacto na longevidade. Os resultados demonstram que a proteína Sit4p regula a via biossintética por um mecanismo que envolve a transcrição dos genes que codificam para as proteínas ceramide sintetase *LAG1* e ceramidase *YPC1*. O rácio (bases esfingóides fosforiladas /bases esfingóides) encontra-se aumentado em células deficientes na proteína Sit4p e o metabolismo é preferencialmente desviado para a síntese de metabolitos de fitoceramida α-hidroxilados, os quais têm sido implicados na regulação da função mitocondrial. Em particular, a diminuição dos níveis totais de ceramida observada no mutante *sit4*Δ está correlacionada com uma activação reduzida da via de sinalização TORC2-Pkh1p-Ypk1p e com a fosforilação diminuída das proteínas Orm. Além disso, a activação da via sinalizadora Pkh1/2p-Sch9p está diminuída nas células deficientes em Sit4p por um mecanismo dependente da proteína Snf1p. A reduzida activação desta via de sinalização está associada com o melhoramento da função mitocondrial e com a extensão da longevidade promovia pela ausência da proteína Sit4p em levedura. Dado o papel regulatório da proteína Sit4p na sinalização mediada por nutrientes e na activação da via sinalizadora Pkh1/2p-Sch9p, os resultados demonstram que a proteína Sit4p se comporta, não só como efectora, mas como um regulador crucial do metabolismo dos esfingolípidos e como tal, está integrada numa rede regulatória de vias de sinalização que actuam de forma coordenada para integrar sinalização derivada de nutrientes transmitida pelo complexo TORC1 com o metabolismo dos esfingolípidos e desta forma regular o crescimento celular e a longevidade.

Em conclusão, o trabalho descrito nesta tese fornece informação adicional em relação aos mecanismos moleculares pelos quais vias de sinalização celulares são activadas em resposta a alterações no metabolismo dos esfingolípidos e o seu impacto em funções celulares cruciais e no metabolismo celular. Tal clarificação é crucial para entender as funções dos esfingolípidos mais detalhadamente e desta forma, definir novas estratégias que contribuam para melhorar a saúde humana e estender a sua longevidade.

CHAPTER I

General Introduction

There is a single light of science, and to brighten it anywhere is to brighten it everywhere.

Isaac Asimov

1.1. Yeast as an eukaryote biological model system

Yeast is an unicellular fungus of the phylum Ascomycetes, class Hemiascomycetes and separated into the main order Saccharomycetales (Guarro *et al.*, 1999). Single cells may undergo budding (eg. *Saccharomyces*) or direct division (fission, eg. *Schizosaccharomyces*) and can even grow as simple irregular filaments (mycelium) (Guarro *et al.*, 1999). In sexual reproduction, most yeasts form asci, which contain up to eight haploid ascospores (Guarro *et al.*, 1999). For the past decades, the budding yeast *Saccharomyces cerevisiae* has been widely used for much of molecular and cellular research because basic aspects of replication, cell division and overall cellular metabolism are highly conserved between yeast and higher eukaryotes, including mammals (Petranovic *et al.*, 2010, Botstein & Fink, 2011). Fundamental processes such as cell cycle (Cross *et al.*, 2011), secretory pathway (Papanikou & Glick, 2009), autophagy (Loewith & Hall, 2011), apoptosis (Carmona-Gutierrez *et al.*, 2010) and the endoplasmic reticulum (ER) unfolded protein response (UPR) (Mori, 2009) have been extensively characterized in yeast. The new insights obtained in yeast have served in many ways to foster our understanding about the role of such cellular processes in human health and disease.

Among all eukaryotic model organisms, *S. cerevisiae* combines several advantages. It can be cultured in different media, it has a short doubling time and convenient experimental tractability, due to simple and inexpensive growth conditions and easy genetic manipulations (Franssens *et al.*, 2013). The complete sequencing of its genome in 1996 was extremely useful for a comprehensive study of genes in human genome and other higher eukaryotes. Furthermore, the ease of genetic manipulation in yeast allows its use for conveniently analyzing and functionally dissecting gene products from other eukaryotes (Franssens *et al.*, 2013). The development of DNA transformation has made yeast particularly suitable for gene cloning and genetic engineering techniques (Winzeler *et al.*, 1999). The identification of genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries (Hu *et al.*, 2007). Plasmids can be introduced into yeast cells either as replicating elements or by chromosomal integration into the genome (Forsburg, 2001, Hu *et al.*, 2007,

Dunham & Fowler, 2013). The phenotypes arising after either deletion or overexpression of yeast genes have significantly contributed to our understanding of proteins function *in vivo*. These techniques have been extensively exploited for the analysis of gene regulation, structure-function relationships of proteins, chromosome structure, and other general questions in cell biology (Forsburg, 2001, Dunham & Fowler, 2013). This is illustrated by the fact that the identification of genes in yeast usually precedes the discovery of human orthologs (approximately 25% of all yeast gene deletions can be complemented by the corresponding human orthologs and 60% of yeast genes have significant homology with at least one conserved domain of human genes) and mammalian genes can be introduced into yeast for systematic analyses of the functions of the corresponding gene products (Foury, 1997, Forsburg, 2001, Smith & Snyder, 2006, Dunham & Fowler, 2013). More recently, heterologous expression of non-natural occurring proteins in yeast has provided novel insights into human age-associated and neurodegenerative diseases, including Alzheimer´s and Parkinson´s diseases (Zhang *et al.*, 1994, Outeiro & Lindquist, 2003, Vanhelmont *et al.*, 2010, Sampaio-Marques *et al.*, 2012, Dunham & Fowler, 2013, Franssens *et al.*, 2013).

As the first available eukaryal genome (http://www.genome.gov/10000510), comprehensive functional, genomic and proteomic methodologies have been firstly established in yeast, which allowed the discovery of a complex array of interacting pathways at a transcriptional and regulatory level (Botstein & Fink, 2011, Dunham & Fowler, 2013). Ultimately, it became the basis of several used technologies, such as yeast two-hybrid, high-throughput screening, microarrays and ChIP (Dunham & Fowler, 2013). Moreover, high-throughput data obtained from functional and genomic tools, such as transcriptome, proteome, metabolome and flux analysis, interactome and locasome analysis are currently compiled in well-curated bioinformatic databases (Botstein & Fink, 2011, Karathia *et al.*, 2011) that provide overall information about protein-protein interactions, genetic interactions, protein function and predicted orthologs in higher eukaryotic organisms (Botstein & Fink, 2011). On this regard, yeast is utterly the eukaryote organism with the most complete experimental database and *S. cerevisiae* has become essential in systems biology by providing a global mapping of gene and protein interaction within the cell and a comprehensive understanding of

eukaryotic cellular processes (Mustacchi *et al.*, 2006).

As any model system, yeast also presents some limitations as a working biological system. One major problem is the fact that yeast is unicellular and much less complex than mammalian cells (Karathia *et al.*, 2011, Zakrajsek *et al.*, 2011). In order to understand the etiology of multifactorial diseases, such as diabetes or metabolic syndrome, studies in yeast are limited due to the absence of multi-organic integration signalling imposed by multicellularity during evolution. For instance, assessing the role of intercellular interactions and systemic mechanisms, such as endocrine and hormone regulation, lies beyond the scope of yeast as a biological system. In neurodegenerative disease, the study of basic aspects associated with synaptic transmission and glial-neuronal processes are hindered (Khurana & Lindquist, 2010). Another important disadvantage is the occurrence of gene duplications, which may difficult the identification of new genes due to redundancy effects (van der Klei & Veenhuis, 2006).

There are also specific aspects related to yeast physiology, such as the cell wall and associated signalling pathways (eg. the CWI pathway) (Levin, 2011). For instance, the lipid and protein composition of cell surface greatly varies between yeast and mammals and therefore response to stimuli may produce distinct effects on cell physiology and metabolism. In pharmacotoxicologic studies, yeast usually require higher concentrations of the compound that is often required to produce a toxic effect in mammalian cell systems, presumably due to the barrier presented by the cell wall and various efflux pumps (Matuo *et al.*, 2012). Importantly, mammalian cells have diversified, from an evolutionary point of view, to include cellular specialization and compartmentalization without homology in yeast. Yeast mitochondria, for example, lack a typical complex I although it possesses a structurally simpler complex named external NADH dehydrogenase 2 (Ndh2p, also known as Nde2p) (Luttik *et al.*, 1998). In this regard, studies assessing the effect of loss-of-function of complex I, such as in Parkinson´s disease (Kosel *et al.*, 1996, Keeney *et al.*, 2006), are limited.

In the scope of this study, the elucidation of sphingolipid metabolism and dynamics and their route of synthesis were primarily done in yeast cells and have been important to uncover new functions of sphingolipids and to understand the mechanisms for sphingolipid homeostasis in human health and disease (Dickson, 2008, Huang *et al.*, 2013). The budding yeast has been used to identify nearly all

of the genes that encode for sphingolipid metabolic enzymes and many of these were critical in identifying mammalian homologs (Rego *et al.*, 2014). Since yeast and mammals share many similarities in sphingolipid metabolism and signalling (Dickson, 2008, Rego *et al.*, 2014), *S. cerevisiae* is considered a valuable model organism to study sphingolipid functions and regulation.

1.2. Aging and disease

Aging is a biological process characterized by a progressive loss of metabolic and physiological integrity of an organism, which leads to a gradual deterioration of biomolecules and impairment of stress resistance mechanisms. Aging has become the primary risk factor for the development of many pathological conditions, including cancer, neurodegenerative diseases, diabetes, cardiovascular disorders, sarcopenia and liver dysfunction (Kirkwood, 2005, Tosato *et al.*, 2007, Newgard & Pessin, 2014).

Several hypotheses have been proposed to explain the etiology of the aging process. It was firstly proposed that aging is stochastic rather than programmed due to a decrease in forces of natural selection for maintenance in the post-reproductive phase of life (reviewed in (Kirkwood, 2008)). From an evolutionary point of view however, biological processes that slow or counteract the deleterious effects of aging are more advantageous and therefore expected to be preferentially selected in individuals to prolong life and give rise to more progeny. As a result of this conception, the notion of gerontogenes (genes that increase lifespan when overexpressed or mutated) and lifespan extension mechanisms have emerged and became the basis for the theory of antagonistic pleiotropy (Williams, 2001, Williams & Day, 2003). It was postulated the existence of positive evolutionary selection of genes in early life stages that confers advantageous effects but possibly adverse effects later in life (at post-reproductive phase) (Williams, 2001, Williams & Day, 2003).

Cells are constantly exposed to a harmful environment throughout life. In particular, early work on DNA damage has established it as a main cause of aging due to gradual impairment of DNA repair processes and DNA loss of integrity (Chen *et al.*, 2007). From this, aging would be genetically determined by deleterious changes on repair and maintenance mechanisms under the genetic control (Chen *et al.*, 2007, Kirkwood, 2008, Rodriguez-Rodero *et al.*, 2011). The disposable soma theory predicts that stochastic accumulated damage and decline of maintenance and repair systems cause aging in somatic tissues, whereas the germ line is carefully maintained and kept immortal (Kirkwood & Holliday, 1979). However, several studies have demonstrated that aging is not fully genetically

programmed and also relies on the interaction with environmental factors, raising to the general notion that aging is a multifactorial phenomenon characterized by a time-dependent decline in physiological function (Kirkwood & Melov, 2011).

Over recent years, the unprecedented advance in genomics and several other ´omic´ areas has provided crucial evidence on the nature and the underlying mechanisms governing the aging process. These approaches include the discovery and identification of key genes and pathways, genetic studies of heritable diseases with common denominators with premature aging and physiological and metabolic profiling that relates cellular and organismal alterations with hallmarks of aging (Mair & Dillin, 2008, Kirkwood & Melov, 2011, Rodriguez-Rodero *et al.*, 2011, Lopez-Otin *et al.*, 2013, Newgard & Pessin, 2014). Importantly, advances in human genetics and cellular biology allowed to scrutinize the sources of aging-related changes, the maintenance and repair mechanisms responsible for cell homeostasis, the outcome resulting from the intersection between deleterious changes and lifespan-promoting processes and finally the ability to ameliorate and retard normal aging (hormetic regulation of aging) and hence extend healthy lifespan. On this regard, aging can be envisioned as a biological process determined by the interconnectedness between genetic and biochemical processes, usually conserved in evolution, and environmental factors. Over a lifetime, this leads to a gradual accumulation of detrimental changes in molecules, cells and tissues associated with a general decline in stress tolerance functions, therefore limiting the ability of an organism to maintain homeostasis. As a result, individuals present higher risk to develop age-related diseases (cancer, cardiovascular and neurodegenerative disorders) and premature mortality (Lopez-Otin *et al.*, 2013).

On the scope of this thesis, some hallmarks of aging will be described, namely genomic and telomere instability, epigenetic alterations, loss of proteostasis, impaired nutrient sensing and stem cell exhaustion.

1.2.1. Genomic instability and telomere shortening

Senescence is a complex process involving genetic and environmental factors affecting most physiological pathways. Among species, the average

lifespan is very wide, which indicates that maximum lifetime is partially determined by specific aspects of species genotypes and ultimately the individual´s genotype.

Over a lifetime, DNA is exposed to several physical and chemical factors (e.g. phthalates and UV radiation) and biological agents (viruses) that compromise genomic stability and integrity (Rodriguez-Rodero *et al.*, 2011). In addition, increased damage promoted by reactive oxygen species (ROS), gradual impairment of DNA repair mechanisms and the accumulation of DNA replication errors during life correlates with cell senescence and aging (Rodriguez-Rodero *et al.*, 2011). It can encompass genomic alterations (point mutations and polymorphisms), loss (or gain) of repeated DNA sequences, telomere shortening, mitogenic signals (oncogenes), gene disruption promoted by transposons and rearrangements in chromosome number (aberrant euploidy and aneuploidy) (Burhans & Weinberger, 2007, Vijg & Suh, 2013).

Importantly, the identification of genes and mutations responsible for age-related monogenic hereditary disorders (progeroid syndromes) has been critical to uncover the function of a specific group of genes in an individual's lifespan. Progerias are characterized by a premature aging phenotype and have become a model to study aging-associated genetic changes (Rodriguez-Rodero *et al.*, 2011). It includes Cockayne syndrome, Werner, Fanconi anemia, Bloom, Rothmund-Thomson and Hutchinson-Gilford syndromes, ataxia-telangiectasia and *xeroderma pigmentosum*, which are characterized by accelerated aging promoted by mutations in genes implicated in genetic stability and DNA repair (Rodriguez-Rodero *et al.*, 2011).

The genomic stability also includes specific mechanisms responsible for the maintenance and functionality of telomeres. Telomeres are DNA-protein complexes that cap and stabilize the ends of linear DNA strands, thus preventing chromosome instability. A correlation between telomere shortening and somatic stem cell decline during aging has been established in the past few decades (Vijg & Suh, 2013, Townsley *et al.*, 2014). It has been shown that repetitive DNA sequences at chromosome ends shortens with age, as observed in fibroblasts and lymphocytes, due to the lack of adequate telomerase activity (Bodnar *et al.*, 1998), but also in hematopoietic stem cells (HSC), making cells more susceptible to mutation, apoptotic cell death and reduced ability to self-renewal (Lansdorp, 1995). In fact, telomere shortening or lengthening is associated with shortened

and extended lifespan in mice, respectively (Bernardes de Jesus *et al.*, 2012). Telomerase deficiency has also been associated with age-associated diseases, such as pulmonary fibrosis, *dyskeratosis congenita* and aplastic anemia, due to replicative senescence and decreased regenerative capacity in tissues (Townsley *et al.*, 2014).

1.2.2. Epigenetic alterations

The emergence of human epigenetics as a key regulator in gene expression and integrity has become the basis for the so-called epigenetic theory of aging (Berdasco & Esteller, 2012). It postulates that non-adaptive epigenetic alterations in cells and tissues contribute to aging. Epigenetic regulation includes alterations in the methylated state of genes and regulatory DNA sequences, covalent modifications of histones, chromatin remodeling and the expression of regulatory non-coding RNAs (Berdasco & Esteller, 2012).

Some studies have demonstrated that epimutations accumulate throughout life, leading to activation of genes normally downregulated epigenetically. In particular, some modifications, such as histone H4K16 acetylation (Dang *et al.*, 2009), H4K20 (Sarg *et al.*, 2002) and H3K4 trimethylation (Greer *et al.*, 2011) and reduced H3K9 methylation or H3K27 (Sidler *et al.*, 2014), have been associated with increased aging. Age-related epigenetic alterations also include the formation of nuclear regions called senescence-associated heterochromatin foci (SAHF) (Narita *et al.*, 2003). At these regions, heterochromatin proteins and Retinoblastoma (Rb) protein are recruited to E2F-dependent promoters of proliferative genes, therefore repressing E2F target genes (Narita *et al.*, 2003).

The activity of DNA methyltransferases, deacetylases and histone demethylases and protein complexes involved in chromatin remodeling is altered with aging (Berdasco & Esteller, 2012). Importantly, the activity of the NAD-dependent deacetylase SIRT1, which belongs to the sirtuin family, decreases throughout life (Berdasco & Esteller, 2012) and Herranz *et al.* have shown that the overexpression of SIRT1 improves healthy lifespan but does not extend longevity (Herranz *et al.*, 2010). Although the mechanisms are rather complex, it affects genome stability by modifying chromatin and repressing the transcription of integrated reporter genes through its intrinsic acetylase activity (Vaquero *et al.*,

2004). Another anti-aging factor that has come to the foreground of research area in recent years is the NAD-dependent deacetylase SIRT6. This protein contributes to genomic stability (DNA repair) and regulates H3K9 and H3K56 deacetylation in telomeric regions, which is involved in NF-kB-dependent modulation of gene expression, apoptosis, and cellular senescence (Kawahara *et al.*, 2009, Yuan *et al.*, 2009). SIRT6-deficient cells are characterized by enhanced genomic instability due to non-clonal chromosomal aberrations and breakage as well as hypersensitivity to γ-irradiation and age-related alterations (cachexia, kyphosis, and osteopenia) (Mostoslavsky *et al.*, 2006), and also the hyperacetylation of the H3K9 locus that enhances NF-kB signalling, which ultimately leads to cell death and aging (Kawahara *et al.*, 2009). On the other hand, increased expression of *SIRT6* prolongs life by mechanisms involving reprogramming of aging cells and alterations in metabolism, such as reduced signalling through the serum insulin-like growth factor (IGF-1) and other members of the IGF signalling pathway (Kanfi *et al.*, 2012), whose activities are known to be lowered in long-lived mutants (Kenyon, 2010).

The DNA methylation pattern of individuals with progerias is altered and resembles the ones in aged cells. More recently, a number of disorders have been documented and associated to genetic imprinting, including cancer and syndromes involving chromosomal instabilities, namely Angelman, Prader-Willi and Beckwith-Wiedemann syndromes (Adams, 2008). These disorders are caused by abnormal activation or silencing of genes by epigenetic mechanisms, usually resulting in non-adaptive alterations of the epigenetic landscape and disease (Adams, 2008, Berdasco & Esteller, 2012).

1.2.3. Loss of protein homeostasis (proteostasis)

During evolution, organisms have developed stress response mechanisms to adapt to harsh environmental conditions and intrinsic stress. In particular, perturbations on protein homeostasis or proteostasis, associated with changes in calcium concentration, glucose deprivation, redox changes or ischemia, impair the function of protein control quality mechanisms and the fidelity of the protein folding (Salminen & Kaarniranta, 2010, Taylor & Dillin, 2011). As a result, the accumulation of unfolded proteins and damaged components has severe

consequences for cell physiology and metabolism and contributes to aging and development of age-related and neurodegenerative diseases, such as Alzheimer´s and Parkinson´s disease (Taylor & Dillin, 2011). This observation is the biochemical basis for a wide variety of diseases termed ER storage or conformational diseases (Rutishauser & Spiess, 2002).

In order to adapt to such hostile environment, the cell has evolved an adaptive and coordinated response to limit the accumulation of unfolded proteins called unfolded protein response (UPR) (Schröder & Kaufman, 2005, Kimata *et al.*, 2006, Mori, 2009). In this mechanism, the ER-located transmembrane protein endoribonuclease IRE1 is autophosphorylated and catalyzes the cytoplasmic splicing of the *XBP1* mRNA. The spliced version of this mRNA is translated into the transcription factor XBP1 that induces various genes to alleviate the ER stress condition (Mori, 2009). At this stage, the folding demand is decreased by downregulating the transcription of genes encoding secretory proteins (Travers *et al.*, 2000), and by promoting the clearance of slowly folding or misfolded proteins through the activation of the ER-associated degradation (ERAD), ubiquitin proteasome system and autophagy (Randolph Y, 2002, Nishikawa *et al.*, 2005, Mori, 2009). Each of these proteostastic mechanisms are capable of eliminating damaged proteins or triggering a more global response, such as cell cycle arrest or apoptosis upon severe proteotoxic stress. To enhance the folding capacity of the ER, the expression of ER resident molecular chaperones and foldases is enhanced (Nishikawa *et al.*, 2005) and the ER increases in size in order to dilute enhanced unfolded protein load, and alleviate the folding capacity and the accumulation of misfolded proteins (Schuck *et al.*, 2009).

The aging process is characterized by the gradual impairment of ER function and proteostasis, eventually leading to the accumulation of harmful protein modifications, misfolding and aggregation of proteins, disturbances in Ca^{2+} homeostasis and impairment in global protein synthesis (Squier & Bigelow, 2000, Kregel & Zhang, 2007, Lindner & Demarez, 2009, Jones, 2010). Moreover, the decline in autophagic and proteasomal degradation with aging compromises protein quality control and promotes the accumulation of misfolded and oxidized proteins. Many of the key components of the UPR such as the chaperones and enzymes display reduced expression and activity, resulting in a dysfunctional ER (Salminen & Kaarniranta, 2010). Unresolved and sustained ER stress leads to a

more deleterious outcome with increased protein accumulation, loss of ER function and activation of apoptotic cascades that ultimately contribute to cell death and aging (Tabas & Ron, 2011).

1.2.4. Impaired nutrient signalling

The ability to utilize substrates and nutrients to fuel energy production for the homeostatic maintenance of cell-intrinsic processes is a characteristic of all living organisms. This is dictated by coordinated nutrient-sensing systems that are able to integrate metabolic and physiological signals to maintain cell and organismal homeostasis. It is well documented that these pathways become increasingly less efficient and dysfunctional, making cells more prone to develop disease. In fact, core metabolic pathways such as glycolysis, fatty acid oxidation, amino acid oxidation, lipogenesis, and ketogenesis become less efficient with aging (Newgard & Pessin, 2014). Crucial sensing protein complexes have received attention in the past few years in the field of aging, namely the nutrient and growth factors sensing mammalian Target Of Rapamycin Complex 1 (mTORC1), the AMP-dependent protein kinase (AMPK) and sirtuins (Newgard & Pessin, 2014).

1.2.4.1. mTORC1 signalling

The mTORC1 is a highly conserved serine/threonine protein kinase complex activated by growth factors, mitogens and nutrients that is involved in the temporal control of cell growth by promoting transcription, ribosome biogenesis, nutrient transport, and inhibiting autophagy (Laplante & Sabatini, 2012, Johnson *et al.*, 2013). The downregulation of mTORC1 activity, either pharmacologically (upon treatment with rapamycin) or genetically, extends the lifespan of several organisms, ranging from yeast (Medvedik *et al.*, 2007, Pan *et al.*, 2011), flies (Bjedov *et al.*, 2010), nematodes (Vellai *et al.*, 2003) alongside with mammals (Harrison *et al.*, 2009, Miller *et al.*, 2011). It also partly mediates the beneficial effects of calorie restriction (CR), which is currently the classic paradigm for studying the role of metabolism in aging (Blagosklonny, 2010, Sharp & Strong,

2010). There is increasing evidence showing that mTORC1 and the protein kinase S6K, one of mTORC1 effectors, contribute to aging and age-related diseases, such as type II diabetes mellitus and cancer (Manning, 2004, Inoki *et al.*, 2005). It has been recently shown that the role of mTORC1-S6K1 signalling in the regulation of longevity is associated with insulin and IGF-1 signalling (Manning, 2004). In mouse models of reduced IGF1 signalling, namely haploinsufficiency of IGF-1 receptor and deletion of insulin receptor substrate-1 (IRS-1), lifespan extension was correlated with reduced mTORC1–S6K1 signalling (Holzenberger *et al.*, 2003, Selman *et al.*, 2008). Moreover, caloric restriction (CR) prolongs lifespan in several models in part due to overlapping effects of reduced mTORC1-S6K1 activity and diminished IGF-1 signalling pathway (reviewed in (Fontana *et al.*, 2010)).

Although the mechanisms involved in the anti-aging effects of downregulation of mTORC1 activity are rather complex and interconnected, some studies have provided evidence that it may promote survival by reducing overall mRNA translation rate and protein synthesis. It would then relieve ER stress, preventing the accumulation of mistranslated and misfolded proteins and loss of proteostasis (Cornu *et al.*, 2013, Johnson *et al.*, 2013). The inhibition of mTORC1 activity also induces macroautophagy (Johnson *et al.*, 2013), which promotes the clearance of damaged biomolecules (DNA, proteins, lipids) and organelles that can be detrimental and induce pathological changes (Cornu *et al.*, 2013, Johnson *et al.*, 2013). mTORC1 inhibition also improves stem cell function and therefore the ability of tissue self-renewal (see 1.2.5.).

1.2.4.2. AMPK signalling

AMP-activated protein kinase (AMPK) is a highly conserved sensor of AMP and ADP levels (Mihaylova & Shaw, 2011). In glucose sensing, AMPK stimulates energy generation from glucose and fatty acids during stress and inhibits energy-consuming processes, such as protein, cholesterol and glycogen synthesis (Hardie, 2007, Mihaylova & Shaw, 2011). Compelling evidence has demonstrated however that the function of AMPK is not restricted to the maintenance of energy metabolism but also in the regulation of several homeostatic mechanisms, such as autophagy (Alers *et al.*, 2012) and general stress response by improving tissue

stress resistance (Greer *et al.*, 2009). For instance, the activation of AMPK reduces oxidative stress by increasing thioredoxin expression (Li *et al.*, 2009) and can suppress deleterious effects promoted by ER stress and inflammation (O'Neill & Hardie, 2013).

The role of AMPK in the regulation of aging process is supported by the general observation that proper energy metabolism is required for adequate organismal homeostasis whereas excessive consumption of energy promotes aging and contribute to pathology. It is therefore not surprising that AMPK activity is increased and mediates some of the anti-aging effects of CR (Canto & Auwerx, 2011). Importantly, the beneficial effects of CR may be related to the downregulation of mTORC1 by AMPK, increased induction of autophagy by inhibiting mTOR and increasing ULK1 signalling and AMPK-induced stimulation of FoxO/DAF-16, Nrf2/SKN-1, and SIRT1 pro-longevity signalling pathways, which improve cellular stress resistance (Salminen & Kaarniranta, 2012). On the other hand, nutritional overload seems to impair AMPK activity and concurrently induce insulin resistance and inflammation in many tissues, thus increasing the propensity to develop obesity, diabetes and cardiovascular diseases (Gauthier *et al.*, 2011).

Several studies have indicated a potentially important role of AMPK orthologues in regulating aging, although the specific mechanisms are not yet fully understood. For instance, loss of the yeast AMPK homolog Snf1p shortens yeast replicative lifespan (Ashrafi *et al.*, 2000). The activation of AAK-2 (AMP-activated kinase-2 in *C. elegans*) and AMPK by metformin or its overexpression extends lifespan (Apfeld *et al.*, 2004, Onken & Driscoll, 2010). The knockout of AMPKα2 has a detrimental effect on health and lifespan in mice. In contrast, metformin treatment in rodents prevents cancer incidence and cardiovascular diseases and extends lifespan (Viollet *et al.*, 2003). More recently, the upregulation of AMPK in the adult *Drosophila* nervous system was shown to induce autophagy both in the brain and the intestinal epithelium and this was linked to reduced IGF-1 signalling and systemic activation of dFOXO, eventually contributing to slow organismal aging (Ulgherait *et al.*, 2014).

The responsiveness of AMPK activation declines during the aging process, presumably due to decreased ability to tolerate metabolic stress. Importantly, the impairment of AMPK signalling has been consistently associated with age-associated diseases, namely cardiovascular diseases and metabolic syndrome.

For instance, Qiang *et al.* reported that impaired AMPK activation and inhibited insulin-stimulated glucose uptake into muscles during aging could enhance the development of metabolic syndrome (Qiang *et al.*, 2007). Turdi *et al.* reported that reduced AMPK activity aggravated aging-driven myocardial dysfunction through impaired mitochondrial function and ROS production (Turdi *et al.*, 2010). Recent associations drawn between the metabolic syndrome and the suppression of AMPK-regulated pathways at a cellular level support that therapeutic activation of AMPK may be beneficial to treat diabetes and obesity (Ruderman *et al.*, 2013). Overall, decreased AMPK activation can decrease cellular autophagy, increase cellular stress and promote inflammation, leading to senescence and aging.

1.2.4.3. Sirtuins

Sirtuins belong to a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases that regulate lifespan in some organisms, including yeast and mice (Nakagawa & Guarente, 2011). The role of sirtuins in aging came from early studies in yeast and worms showing that the deletion of *SIR2* gene severely shortens lifespan in *S. cerevisiae*, while its overexpression extends lifespan (Kaeberlein *et al.*, 1999). Moreover, the sirtuins activator resveratrol mimics CR by enhancing Sir2p activity, which in turn increases DNA stability and promotes yeast lifespan extension by 70% (Howitz *et al.*, 2003). The overexpression of Sir-2.1, the homologue of yeast Sir2p, increases lifespan in *C. elegans* and *Drosophila*. Importantly, Banerjee *et al.* found that deletion of *dSir2* abrogates the lifespan-extending effect by starvation, demonstrating that dSir2 expression is required for longevity in starvation conditions (Banerjee *et al.*, 2012). More recently, an interplay between sirtuins, ROS signalling and aging has emerged (Merksamer *et al.*, 2013). Upon activation, the (NAD+)-dependent deacetylases SIRT1 and SIRT3 deacetylate some proteins, namely superoxide dismutase SOD2, and increase the expression of oxidative stress genes including glutathione peroxidase (*GPx1*), catalase, and manganese SOD (MnSOD), which mediate some beneficial effects of CR by decreasing ROS levels and oxidative damage (Merksamer *et al.*, 2013).

The importance of SIRT1 is demonstrated by the observation that a high-fat diet leads to cleavage of SIRT1 induced by inflammation in mice (Chalkiadaki &

Guarente, 2012), and obesity produces similar effects in humans (Pedersen *et al.*, 2008). Similarly, loss of sirtuin activity contributes to metabolic syndrome and diabetes in mice and humans whereas transgenic overexpression of SIRT1 attenuates the onset of these age-related diseases. For instance, the mild overexpression of SIRT1 in mice on a high-fat diet and diabetic mice causes a significant reduction in blood glucose and plasma insulin levels (Li *et al.*, 2011). The protective effect of SIRT1 also appears to be related to the mitigation of pro-inflammatory responses, since the overexpression of SIRT1 diminishes hepatic steatosis and adipose tissue–specific inflammation (Li *et al.*, 2011). In addition, histone deacetylation has been shown to decrease the induction of pro-inflammatory genes (Nakagawa & Guarente, 2011) and SIRT1 seems to play an important role. In fact, decreased SIRT1 levels are linked with the onset and progression of inflammatory-associated disease and the activation of SIRT1 may prevent acute events following induction of inflammation (Nakagawa & Guarente, 2011).

Another sirtuin protein involved in aging is SIRT6. Mice deficient in SIRT6 exhibit severe metabolic defects and premature aging (Mostoslavsky *et al.*, 2006), whereas the overexpression of *SIRT6* promotes longevity in male mice (Kanfi *et al.*, 2012). Conversely, *SIRT6* overexpression protects from obesity with enhanced glucose tolerance and reduced fat storage (Kanfi *et al.*, 2010). The effect seems to be, in part, mediated by the downregulation of the IGF-1 signalling pathway, which contributed to extend lifespan in male mice (Kanfi *et al.*, 2012). Nevertheless, the protective effect of SIRT6 (and SIRT1) could also be related to DNA damage response, genome integrity and telomere maintenance, as previously discussed (Nakagawa & Guarente, 2011) (see 1.2.2.).

1.2.5. Stem cell exhaustion

Aging is determined by alterations in differentiated cells, such as neurons, and partly by changes in mitotic cells, including stem cells and restricted progenitors. Stem cells persist throughout life in numerous mammalian tissues and contribute to maintain organismal homeostasis due to the regenerative role in tissues. However, the decline in stem cell function with aging is well documented, including in blood cells, forebrain, skeletal muscle, and skin cells (Lopez-Otin *et*

al., 2013, Oh *et al.*, 2014). Therefore, age-related changes in the function of stem cells and other progenitors may contribute to some aspects of disease and aging.

The attrition of stem cell regenerative capacity is usually associated with DNA damage, telomere shortening and mitochondrial damage with aging (Oh *et al.*, 2014). These are examples of how the decline of stem cell function emerges as an integrative consequence of overall cellular damage in the organism. In fact, mitochondrial activity, tissue growth, and metabolic rates during development influence the rates of cellular growth and proliferation and stem cells must be able to adapt their function to match the demands of tissues throughout life (Lopez-Otin *et al.*, 2013). Therefore, stem cell quiescence during aging derives from the interactions between cell-intrinsic (proliferation, frequency, differentiation) and cell-extrinsic mechanisms (nutrient sensing, inflammation) (Oh *et al.*, 2014).

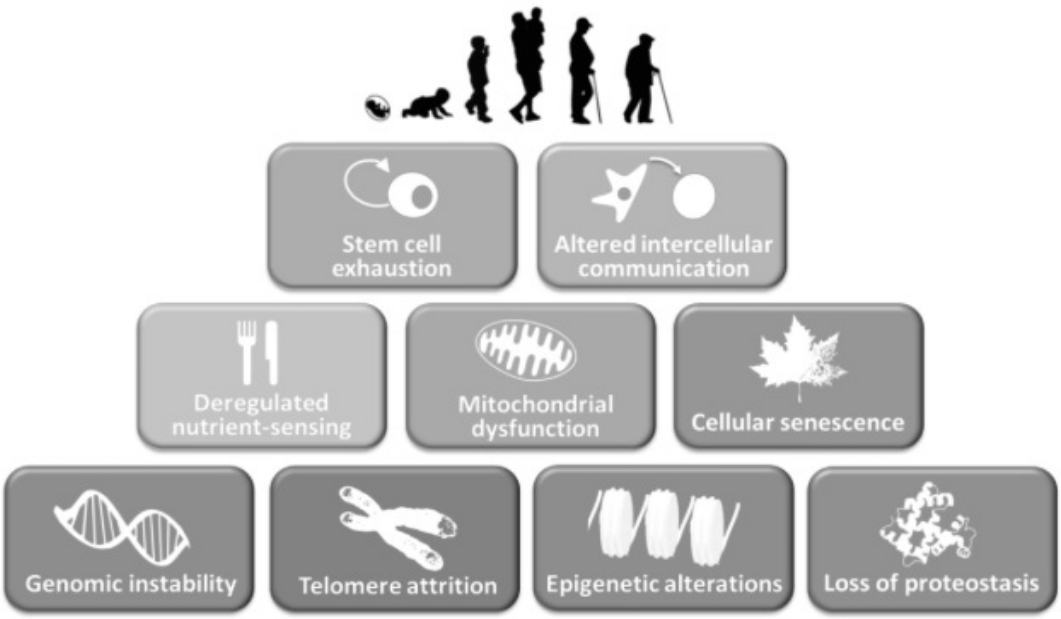


Figure 1.1. **The hallmarks of aging**. The main causes of the aging process are depicted and currently the major challenge is to establish the interconnection between these hallmarks and define new strategies to improve healthy lifespan and prevent the onset of age-related diseases, such as cancer, neurodegenerative, cardiovascular, liver and inflammatory diseases. The Figure was adapted from López-Otín *et al.*, 2013.

Importantly, some anti-aging interventions, such as CR or administration of rapamycin (inhibition of mTOR), have been shown to improve stem cell aging in the epidermis, hematopoietic system and intestine (reviewed in (Ramos &

Kaeberlein, 2012)). Parabiosis experiments, which involve the exposure to systemic and humoral factors from a young parabiont (sharing circulation with an old mouse), also proved to restore proper stem cell function (Conboy *et al.*, 2013).

Taking altogether, aging is dictated by the impairment of several genetic and cellular mechanisms and disruption of signalling pathways with time that culminates in the progressive inability to maintain organismal homeostasis and the emergence of age-related disease and premature mortality (Figure 1.1).

1.3. Oxidative stress and mitochondrial dysfunction in the aging process

1.3.1. The mitochondrial free radical theory of aging

Mitochondria are eukaryotic organelles involved in various functions, namely amino acid and nitrogen metabolism, apoptosis, ion and metabolite flux, intermediate metabolism (Krebs cycle), lipid metabolism, nucleic acid metabolism, mitochondrial DNA (mtDNA) inheritance, signal transduction, stress response and most importantly ATP production through the respiration chain/oxidative phosphorylation process (Hajnoczky & Hoek, 2007, Pereira *et al.*, 2008, Vendelbo & Nair, 2011). The mitochondrial matrix is the site of metabolic processes such as the Krebs cycle, fatty-acid oxidation, Fe-S biogenesis, and heme synthesis (Pereira *et al.*, 2008, Lill *et al.*, 2012). Mitochondrial protein folding and degradation (mitochondrial unfolded protein response, mtUPR), mtDNA replication, transcription and translation also occur in the matrix (Pellegrino *et al.*, 2013). The inner membrane contains protein complexes necessary for electron transport and ATP synthesis, metabolite transporters, and factors involved in mitochondrial turnover. Cytochrome *c* is the most abundant component in the intermembrane space, which also includes mitochondrial fusion proteins, import proteins and redox enzymes (Westermann, 2010, Pellegrino *et al.*, 2013). The outer membrane includes protein transporters, and factors involved in metabolite influx/efflux, mitochondrial fission and fusion and apoptosis (Pereira *et al.*, 2008, Westermann, 2010).

The recognition of the importance of oxidative stress in longevity pathways has become the hallmark of the free radical theory of aging firstly proposed by Harman in 1956 (Harman, 1956). It is proposed that cumulative damage to biological macromolecules by ROS leads to irreversible cell damage and overall functional decline. Later on, the free-radical theory was refined in 1972 to include mitochondria, as they constitute a major site for ROS production (Harman, 1972).

ROS are byproducts formed resulting from the electron leakage in complex I and complex III, where oxygen is reduced to arise the superoxide radical. Superoxide dismutases convert superoxide radicals into hydrogen peroxide, which

can be further reduced to hydroxyl radicals by Fe^{2+}. All together, these ROS are key contributors to oxidative damage (Vendelbo & Nair, 2011) (Figure 1.2). Numerous studies provide evidence for the impact of oxidative imbalance in aging, mostly using animal models.

The involvement of mtDNA in longevity pathways has received considerable attention (Shokolenko *et al.*, 2014). Indeed, it presents a higher mutation rate, which is attributed to its close proximity to the electron transport chain. Unlike nuclear DNA, mtDNA does not present the protective effect of histones against oxidative damage but instead forms protein-DNA complexes (nucleoids) in the mitochondrial matrix (Shokolenko *et al.*, 2014). Although this nucleoid formation makes mtDNA more impervious to oxidative damage, it remains more susceptible to ROS-mediated damage than nuclear DNA (Shokolenko *et al.*, 2014).

During lifetime, the random accumulation of age-related somatic mtDNA mutations contributes to aging (Shokolenko *et al.*, 2014). However, an alternative hypothesis has emerged, claiming that most of the mutations are created as replication errors during embryogenesis and then undergo clonal expansion and cause a mosaic respiratory chain dysfunction in different tissues. Mosaic respiratory chain deficiency caused by clonal expansion of mtDNA mutations is ubiquitously observed in human aging (Larsson, 2010). In an elegant work, Bonawitz *et al.* demonstrated that in yeast expressing a deficient mitochondrial RNA polymerase amino-terminal domain (ATD) mutant (which has efficient but imbalanced mitochondrial translation), defective coupling of transcription to translation leads to increased ROS production and inhibition of respiration in the stationary phase, ultimately limiting CLS (Bonawitz *et al.*, 2006).

Therefore, life-long exposure to ROS likely results in a preferential accumulation of mtDNA damage and accelerated aging. Nowadays, the refined mitochondrial free radical theory of aging states that the mitochondrial production of ROS, such as superoxide and H_2O_2, results in the accumulation of damage to macromolecules and stress resistance protein systems, which in turn overwhelm the capacity of biological systems to repair themselves, resulting in an inevitable functional decline. The cumulative increase of ROS production and aging-associated mutations and alterations in mtDNA stability and integrity can impair the function of the respiratory chain and enhance ROS production. This can

subsequently leads to exponentially increasing levels of mtDNA damage and oxidative stress in the cell, which ultimately culminates in cell death and aging (Shokolenko *et al.*, 2014).

More recently, the mitochondrial free radical theory of aging has been challenged. Some recent studies have demonstrated that altered ROS signalling can either have no significant or can even be beneficial for longevity. For instance, increased ROS production can extend lifespan in yeast (Pan *et al.*, 2011) and *C. elegans* (Lee *et al.*, 2010). In yeast, enhanced ROS levels during growth produce a hormetic effect and activate adaptive stress response mechanisms, pre-conditioning cells to better survive the stationary phase and to extend CLS (Pan *et al.*, 2011).

1.3.2. ROS scavenging systems

To live in an oxygen-containing environment, organisms had to evolve efficient antioxidant defense systems to cope with the production of ROS. Such mechanisms comprise either non-enzymatic and enzymatic components to scavenge ROS. ROS-scavenging pathways from different cellular compartments act coordinately to avoid the deleterious effects of toxic oxygen products and oxidative damage to lipids, proteins, and nucleic acids.

1.3.2.1. Non-enzymatic components of the antioxidant defense system

I. Glutathione

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is a low molecular weight thiol with an important regulatory role against oxidative damage (Davies, 2000) (Figure 1.2). It has been detected virtually in all cellular compartments such as cytosol, ER, lysosome, and mitochondria. Due to the presence of a free thiol group that can transfer two electrons, it can effectively neutralize ROS. During ROS scavenging, two molecules of glutathione are oxidized to render glutathione disulfide (GSSG) (Davies, 2000, Circu & Aw, 2012). The balance between GSH and GSSG is crucial to maintain cell´s redox state and the activity of glutathione reductase, a NADPH-dependent enzyme, is required to

preserve this balance (Davies, 2000, Circu & Aw, 2012). Due to its reducing power, GSH plays an important role in diverse biological processes, including cell growth/division, signal transduction, conjugation of metabolites, enzymatic regulation, synthesis of proteins and nucleic acids, detoxification of xenobiotics and the expression of stress-responsive genes (Aquilano *et al.*, 2014). This relevance is supported by the fact that glutathione exist in cells at high concentrations (1-10 mM) (Circu & Aw, 2012).

II. Natural Antioxidants: Vitamins and Polyphenols

Diet is an important non-genetic factor for the control of some diseases. More specifically, fruits and vegetables have been reported to have a protective effect due to high content of vitamins A, C, and E and polyphenol, which present antioxidant activity (Landete, 2013). Due to their ability to scavenge ROS, they slow or prevent the oxidation of other molecules by removing free radical intermediates and inhibiting other oxidation reactions (Landete, 2013). Although some inconsistencies on the importance of exogenous antioxidants *in vitro* and *in vivo* have been reported, it is generally accepted that endogenous homeostatic repair mechanisms are unable to prevent global oxidative damage, particularly with aging, thereby making sources of dietary antioxidants especially important to ameliorate the impact of cumulative oxidative stress overtime (Landete, 2013).

1.3.2.2. Enzymatic components of the antioxidant defense system

I. Superoxide dismutase (SOD)

Superoxide dismutase plays a central role in defense against oxidative stress in all aerobic organisms. The enzyme SOD belongs to the family of metalloenzymes and catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2 (Figure 1.2) (Davies, 2000, Fukai & Ushio-Fukai, 2011). It is present in most of the subcellular compartments that generate activated forms of oxygen. In eukaryotic cells, three isozymes of SOD are known, namely SOD1, a copper/zinc SOD (Cu/Zn-SOD), SOD2, a manganese SOD (Mn-SOD), and SOD3, an extracellular SOD (EC-SOD) that contains copper and zinc (Fukai & Ushio-Fukai, 2011). Mn-SOD is localized in

mitochondria, whereas SOD1 is found in the cytosol, and peroxisome and mitochondria (Fukai & Ushio-Fukai, 2011). *S. cerevisiae* possesses a Cu/ZnSOD (Sod1p), predominantly cytosolic, and a mitochondrial form of Mn-SOD (Sod2p) (Gralla, 1997).

Superoxide anions can also be eliminated by metallothioneins, which are small and ubiquitous Cys-rich proteins known to be involved in ROS scavenging and metal homeostasis (Babula *et al.*, 2012).

II. Catalase and peroxidases

Among antioxidant enzymes, catalase was the first enzyme to be identified and characterized in detail. It is a ubiquitous tetrameric heme-containing enzyme that catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen (Davies, 2000) (Figure 1.2). It has high specificity for H_2O_2, but weak activity against organic peroxides. Catalases break down hydrogen peroxide by a two-stage mechanism in which hydrogen peroxide alternately oxidizes and reduces the haem iron at the active site. In the first step, one hydrogen peroxide molecule oxidizes the haem to an oxyferryl species. In the second step, a second hydrogen peroxide molecule is used as a reductant to regenerate the enzyme, producing water and oxygen (Gralla, 1997). Catalase is located in the cytosol and the matrix of peroxisomes and mitochondria in eukaryotic cells (Gralla, 1997). Interestingly, alterations in catalase subcellular localization have been observed in association with disease and stress conditions (Zhou & Kang, 2000). In *S. cerevisiae*, two forms of catalase exist, namely the cytosolic catalase Ctt1p and the peroxisomal/mitochondrial catalase Cta1p (Gralla, 1997).

Glutathione peroxidase belongs to the peroxidase family whose main biological role relies on the ability to prevent oxidative stress by reducing lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water (Arthur, 2000, Circu & Aw, 2012, Aquilano *et al.*, 2014). Several isozymes were identified in mammals and are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant form of GPx and is mostly located in the cytosol of virtually any mammalian tissue (Figure 1.2).

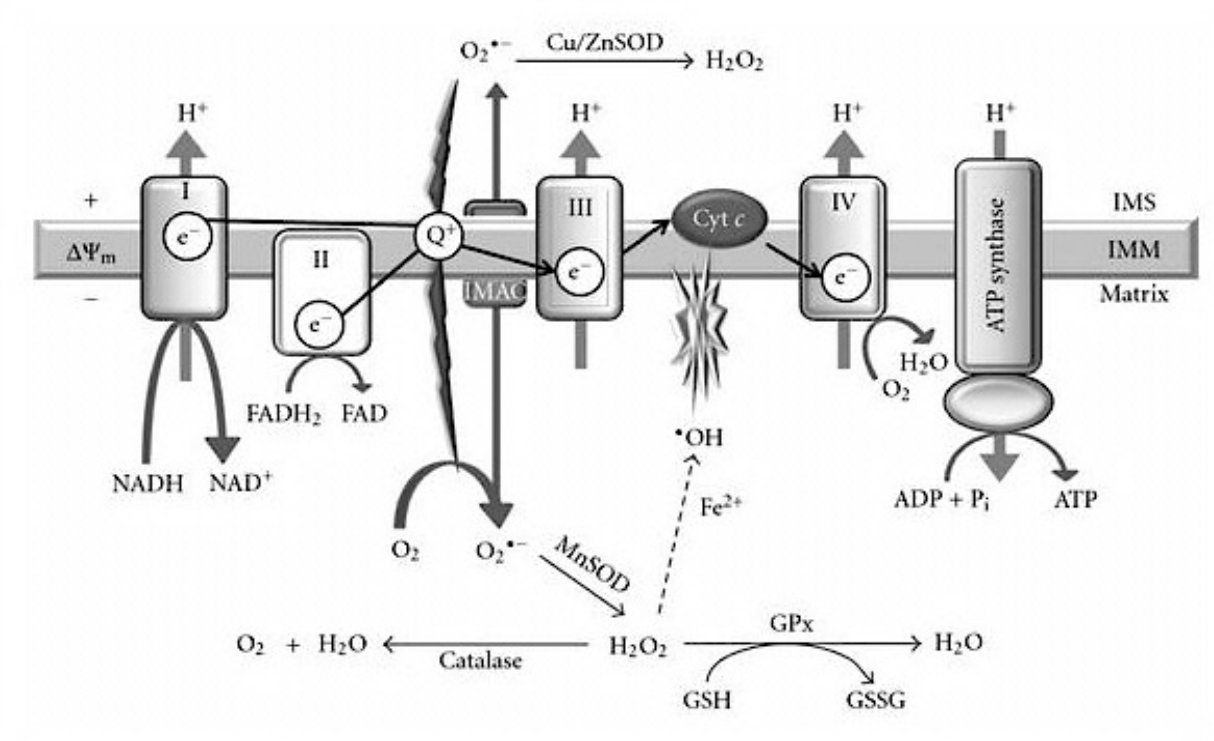


Figure 1.2. Cellular ROS production. Mitochondria (and ER) constitute the major source of ROS production in cells. The reduction of oxygen to water through the respiratory chain proceeds via one electron flow. In the mitochondrial respiratory chain, Complex IV (cytochrome *c* oxidase) retains all partially reduced intermediates until full reduction is achieved. However, the redox centers in the electron transport chain may leak electrons to oxygen, partially reducing this molecule to superoxide anion ($O_2^{\bullet-}$). Dismutation of $O_2^{\bullet-}$ produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH•) by the Fenton reaction. Several antioxidant defense mechanisms contribute to attenuate oxidative damage to biomolecules, namely glutathione (GSH), glutathione peroxidases (GPx), catalase and superoxide dismutase (SOD).

Glutathione peroxidase 2 (GPx2) is an gastrointestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially abundant in the plasma. Eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans and they require selenium for optimal catalytic and antioxidant activity (Arthur, 2000).

1.3.3. Yeast as a model to study the role of mitochondria in aging

S. cerevisiae has been crucial to unravel the mechanisms by which cells respond to mitochondrial dysfunction and oxidative stress. This involves functional and structural alterations in mitochondrial morphology and turnover in response to metabolic and environmental cues elicited by cellular signalling pathways. In this section, the importance of general cell signalling pathways and mitochondrial quality control mechanisms (mitochondrial dynamics, macroautophagy and mitophagy) will be introduced as an emerging link to the aging process.

1.3.3.1. TORC1-Sch9p and RAS/PKA pathways in the regulation of mitochondrial function, stress response and aging

Aging in yeast is monitored by counting the number of daughter cells generated by an individual mother cell (replicative lifespan, RLS) or by evaluating the survival of a population of post-mitotic cells (chronological lifespan, CLS) (Fabrizio & Longo, 2003). In *S. cerevisiae*, glucose, aminoacids and other nutrients can activate the responsive Target of Rapamycin (TOR) and the serine threonine kinase Sch9p and the Ras/PKA signalling pathways (Fontana *et al.*, 2010) with significant impact on longevity.

An important signalling pathway involved in the regulation of cell growth and survival is the highly conserved TOR pathway (Dann & Thomas, 2006). This pathway is highly conserved among organisms, ranging from flies, nematodes, protozoa alongside with mammals. In *S. cerevisiae*, the TOR pathway is controlled by two Ser/Thr protein kinases, Tor1p and Tor2p, which assemble into two protein complexes with distinct subunit composition and regulatory roles (Loewith *et al.*, 2002, Dann & Thomas, 2006, Kim & Guan, 2011, Loewith & Hall, 2011). The rapamycin-sensitive TOR complex 1 (TORC1) contains either Tor1p or Tor2p and is mostly associated with the regulation of cell growth (nutrient sensing), autophagy, ribosomal and protein turnover and cell proliferation (Evans *et al.*, 2011, Kim & Guan, 2011). The TOR complex 2 (TORC2) contains Tor2p, but not Tor1p, and mediates the proper maintenance of the cell cytoskeleton (Cybulski & Hall, 2009) and was recently implicated in the regulation of ceramide biosynthesis

by a Ypk2p-dependent mechanism (Aronova *et al.*, 2008). Furthermore, TORC2 indirectly modulates the sphingomyelinase Isc1p activity through the phosphorylation and activation of Slm1p and Slm2p (Tabuchi *et al.*, 2006).

Some studies have linked the TORC1 pathway to the regulation of mitochondrial function and yeast CLS (Bonawitz *et al.*, 2007, Pan *et al.*, 2011). In fact, the deletion of *TOR1* or pharmacological inhibition of TORC1 with rapamycin promote oxidative stress resistance and extends CLS in yeast and other organisms (Powers *et al.*, 2006, Bonawitz *et al.*, 2007, Kaeberlein & Kennedy, 2011) (Figure 1.3). TORC1 is active during early stages of growth and represses the induction of stress responses and entry into the stationary phase, in part by inhibiting the Rim15p protein kinase (Wanke *et al.*, 2005, Wei *et al.*, 2008). Reducing TORC1 signalling at early stages of growth extends CLS by an intrinsic mechanism involving enhanced mitochondrial membrane potential and superoxide production (Pan *et al.*, 2011). This in turn induces an adaptive response that contributes to decrease ROS production in the stationary phase and promote longevity in yeast. Associated with improved mitochondrial function, *TOR1* deletion leads to increased translation of mtDNA-encoded subunits of the oxidative phosphorylation system, which increases oxygen consumption and may limit intracellular oxygen availability to produce ROS (Bonawitz *et al.*, 2007). As observed in yeast, administration of rapamycin leaded to the downregulation of the mTOR-S6K pathway and reduction in ROS levels and oxidative DNA damage in mammalian cells (Halicka *et al.*, 2012).

Some authors have identified downstream targets of TORC1 involved in the regulation of stress response and aging, namely the AGC protein kinase Sch9p. Like other AGC proteins, Sch9p has several conserved domains: a central kinase catalytic domain, an activation loop, a turn motif (TM) and a C-terminal regulatory domain, which contains a hydrophobic motif (HM) that is phosphorylated by TORC1 (Urban *et al.*, 2007). At the N-terminal side of the activation loop, Sch9p has a calcium-dependent C2 domain with unknown function (Urban *et al.*, 2007). Sch9p acts as a signalling mediator, relaying upstream signals from intracellular and extracellular cues, to downstream targets by phosphorylating them on serine and/or threonine residues (Voordeckers *et al.*, 2011, Halicka *et al.*, 2012, Huang *et al.*, 2012).

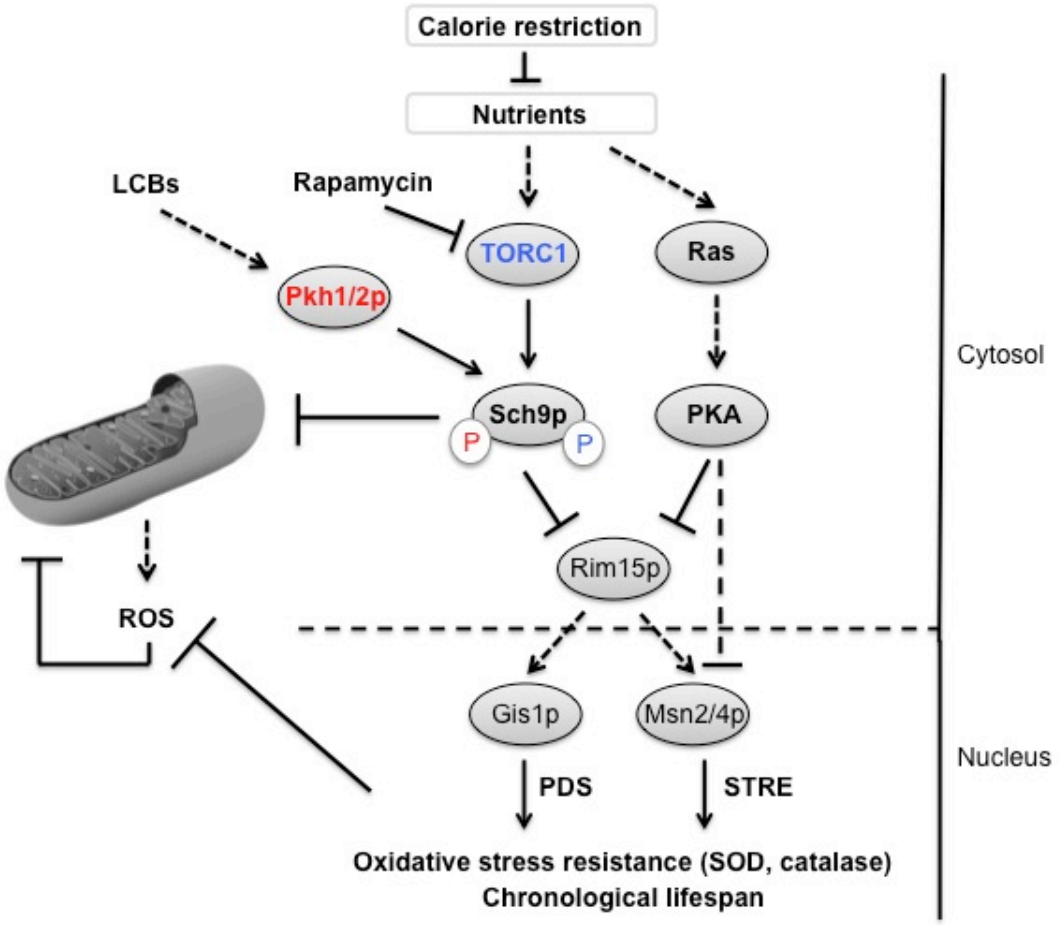


Figure 1.3. The yeast major regulatory signalling pathways of CLS and mitochondria function. The nutrient-sensing pathways controlled by TORC1, Sch9p and Ras/PKA converge on the protein kinase Rim15p, which in turn regulates the activation of Msn2/4p and Gis1p stress-responsive transcription factors. Pkh1/2p also regulates Sch9p activity in response to sphingolipids (long chain sphingoid bases, LCBs). The downregulation of Ras/PKA and TORC1-Sch9p pathways partly mediate the anti-aging effects of CR.

Sch9p has a pivotal role in oxidative stress resistance, CLS and mitochondrial function. In fact, the deletion of *SCH9* gene leads to improved mitochondrial function, which contributes to oxidative resistance and CLS extension in yeast (Fabrizio *et al.*, 2001, Wei *et al.*, 2008, Wei *et al.*, 2009) (Figure 1.3). Apart from sensing nutrient and stress signals from TORC1, Sch9p also regulates CLS and mitochondria function by integrating signals from long chain sphingoid bases (LCBs), a sphingolipid species (Figure 1.3). In addition to phosphorylation in the C-terminus mediated by TORC1, Sch9p is phosphorylated in a Thr570 residue in the activation loop by Pkh1/2p protein kinases, homologs of

mammalian phosphoinositide-dependent protein kinase 1 (PDK1), in response to LCBs (Voordeckers *et al.*, 2011, Huang *et al.*, 2012). Huang *et al.* have recently demonstrated that the downregulation of sphingolipid synthesis induced by myriocin (an inhibitor of the first step of *de novo* sphingolipid biosynthetic pathway) or the deletion of *PKH2* enhances CLS and improves mitochondrial function and oxidative stress resistance by Sch9p-dependent mechanisms, which involves a decrease in the activation of the Pkh1/2p-Sch9p pathway (Huang *et al.*, 2012).

Another important signalling pathway in the regulation of mitochondrial function and CLS in yeast is the Ras/PKA pathway. *S. cerevisiae* contains 2 *RAS* genes, *RAS1* and *RAS2*, which present significant homology to the mammalian Ras. Both Ras1p and Ras2p function to activate adenylate cyclase (Tamaki, 2007). Adenylate cyclase converts ATP into cAMP, which then binds the Bcy1p protein, a regulatory subunit of protein kinase A (PKA) (Tamaki, 2007). Three genes, *TPK1*, *TPK2*, and *TPK3*, encode the catalytic subunit of protein kinase A. When cAMP is hydrolyzed by phosphodiesterases encoded by the *PDE1* and *PDE2* gene, PKA is inactivated due to binding of Bcy1p to the catalytic protein complex (Tamaki, 2007).

The TORC1-Sch9p and the RAS/cAMP/PKA pathways regulate mitochondrial function and longevity by overlapping mechanisms (Wei *et al.*, 2008, Galdieri *et al.*, 2010) (Figure 1.3). In fact, the downregulation of both pathways extends longevity and increases stress resistance partly through the Rim15p protein kinase (Wei *et al.*, 2008, Wei *et al.*, 2009). Rim15p is inhibited by Sch9p, Ras/PKA and TORC1 during growth. Mechanistically, inactivation of TORC1 results in dephosphorylation of the phospho-Thr1075 located in a 14-3-3-binding site of Rim15p, thereby causing the release of Rim15p from the cytoplasmic 14-3-3 anchoring protein Bmh2p (Galdieri *et al.*, 2010). For the remaining signalling effectors (PKA and Sch9p) involved in the inactivation of Rim15p, the mechanism is not yet known.

When these signalling pathways are downregulated, Rim15p becomes activated and increases the expression of a variety of genes involved in G_0 entry and stress response through the translocation of transcription factors Msn2/4p and Gis1p into the nucleus (Wei *et al.*, 2008, Galdieri *et al.*, 2010) (Figure 1.3). The core of the post diauxic shift (PDS)-element and stress responsive element (STRE)-element genes, whose expression is induced by these transcription

factors, are important in response to a variety of stresses, including nutrient limitation and oxidative stress and also in trehalose and glycogen accumulation, which are required to survive under nutrient starvation (during stationary phase) and longevity in yeast (Galdieri *et al.*, 2010). The transcription factors Msn2p/Msn4p upregulate genes encoding for antioxidant defense mechanisms, including *SOD2*, *CTT1*, *SOD2*, *GLR1*, *HSP12* and *DDR2*, as well as the nicotinamidase *PCN1* (Jamieson, 1998, Zhang *et al.*, 2009, Galdieri *et al.*, 2010). Pcn1p is involved in the NAD salvage pathway and favors Sir2p activation to suppress the formation of toxic replicative ribosomal DNA (rDNA) (Lesur & Campbell, 2004). Gis1p-regulated genes include stress response (*HSP26*, *SIP18* and *GRE1*), the pentose phosphate pathway (*TKL2*, *GND2*), glutamate synthesis (*IDP2, GDH3*) and the glyoxylate cycle (*MLS1*, *ICL1*) genes (Zhang *et al.*, 2009).

Moreover, Msn2/4p and Rim15p are required for the induction of autophagy upon inhibition of PKA and Sch9p, although they are dispensable in the induction of autophagy by rapamycin (Yorimitsu *et al.*, 2007). It was also shown that the upregulation of Rim15p is required to improve oxidative stress response and mitochondrial function and to extend yeast lifespan in CR conditions downstream of Ras/PKA, TORC1 and Sch9p pathways (Wei *et al.*, 2008). More recently, it was shown that aminoacids threonine and valine promote increased sensitivity to general stress and aging primarily by activating the TORC1-Sch9p pathway whereas serine stimulates Sch9p through Pkh1/2p, which in turn inhibits Rim15p activity (Mirisola *et al.*, 2014).

1.3.3.2. Mitochondrial quality control mechanisms

Mitochondrial dynamics, which is dictated by a series of fusion and fission events in the mitochondrial network, is involved in the regulation of important cellular processes, namely mitochondrial metabolism, redox dynamics, apoptosis and cell death. Together with macroautophagy and the selective mitochondrial turnover (mitophagy), mitochondrial dynamics forms an interconnected quality control system that contributes to homeostatic turnover of mitochondria and sustained ATP production and removal of damaged mitochondria and oxidized biomolecules (proteins, DNA and lipids), processes that are tightly regulated to preclude aging. It is therefore conceivable that dysfunctional quality control

mechanisms may constitute a major cause of mitochondrial dysfunction, leading to oxidative stress and cell death during aging.

1.3.3.2.1. Mitochondrial dynamics

Mitochondria are dynamic structures that migrate throughout the cell, fuse and divide, and undergo regulated turnover. These highly coordinated processes are important on the regulation of mitochondrial function and cell physiology by allowing mitochondrial compartmentalization within the cell, mitochondrial communication, regulation of the mitochondrial shape and to general quality control (Westermann, 2010).

Mitochondrial morphology relies on a fine balance between fusion and fission activities. The induction of mitochondrial fusion allows the formation of extended and interconnected mitochondrial networks, whereas a shift toward fission generates numerous morphologically and functionally distinct organelles, suggesting that this morphological adaptation is important for mitochondrial adaptation and compartmentalization according to cellular demands (Detmer & Chan, 2007, Berman *et al.*, 2008, Chen & Chan, 2009, Westermann, 2012, Mirisola *et al.*, 2014) (Figure 1.4). Furthermore, the connectivity of the mitochondrial network is an important factor that determines the cell's response to calcium signals, which is an important pro-apoptotic factor (Westermann, 2010).

The fusion of mitochondria is an essential step in certain developmental processes, such as embryonic development and spermatogenesis (Zhang *et al.*, 2007). In addition to mitochondrial network formation, fusion serves to mix and unify the mitochondrial compartment, which is thought to constitute an adaptive mechanism against gradual accumulation of damage and mtDNA deterioration with aging (Zhang *et al.*, 2007, Westermann, 2010). Apparently, fusion of mitochondria may counteract deleterious biological repercussions for the cell by allowing complementation of mtDNA gene products in heteroplasmic cells that have accumulated different somatic mutations, thus diluting the effect of deleterious mtDNA mutations and depolarized mitochondria during aging (Zhang *et al.*, 2007, Seo *et al.*, 2010, Westermann, 2010).

The major components of the mitochondrial fusion machinery have been evolutionarily conserved from yeast to mammals. The protein machinery has been primarily identified in the yeast *S. cerevisiae*, which has emerged as the prime model organism to study the molecular mechanisms of mitochondrial membrane fusion (Westermann, 2008). The fusion core machinery in yeast consists of three proteins, namely Fzo1p (the yeast homologue of mammalian mitofusins MFN1 and MFN2) and Ugo1p (orthologs in higher organisms were not identified), which are located in the outer membrane. Mgm1p, the yeast homologue of OPA1 in mammals, is an intermembrane space protein anchored to the inner membrane (Westermann, 2008, Seo *et al.*, 2010) (Figure 1.4). Mutant yeast cells on these proteins are characterized by a fragmented mitochondria phenotype and defective mtDNA inheritance, presumably due to unopposed fusion (Seo *et al.*, 2010, Westermann, 2012). Exchange of mitochondrial matrix content is blocked both *in vivo* and *in vitro*, suggesting that these proteins are required for mitochondrial fusion. In this process, Fzo1p mediate outer membrane fusion whereas Mgm1p has the ability to form trans-complexes that tether apposing inner membranes (Figure 1.4) (Westermann, 2010). Studies in mammals have demonstrated that OPA1 is the main mediator of inner membrane fusion and maintenance of mtDNA integrity (Song *et al.*, 2009).

The relevance of mitochondrial fusion to underlying mechanisms of aging has recently been highlighted. Mutations in MFN2 impair oxidative metabolism, cell-cycle progression and cell survival, and contribute to Charcot-Marie-Tooth neuropathy type 2A (Chen & Chan, 2009). Absence of MFN1 and MFN2 promotes mitochondrial fragmentation and enhanced sensitivity to apoptotic stimuli (Sugioka *et al.*, 2004). In contrast, overexpression of *MFN1/2*, apart from increasing mitochondrial connectivity, delays Bax activation, cytochrome *c* release, and apoptotic death, implicating mitofusins in cell death (Suen *et al.*, 2008, Westermann, 2010) (Figure 1.4).

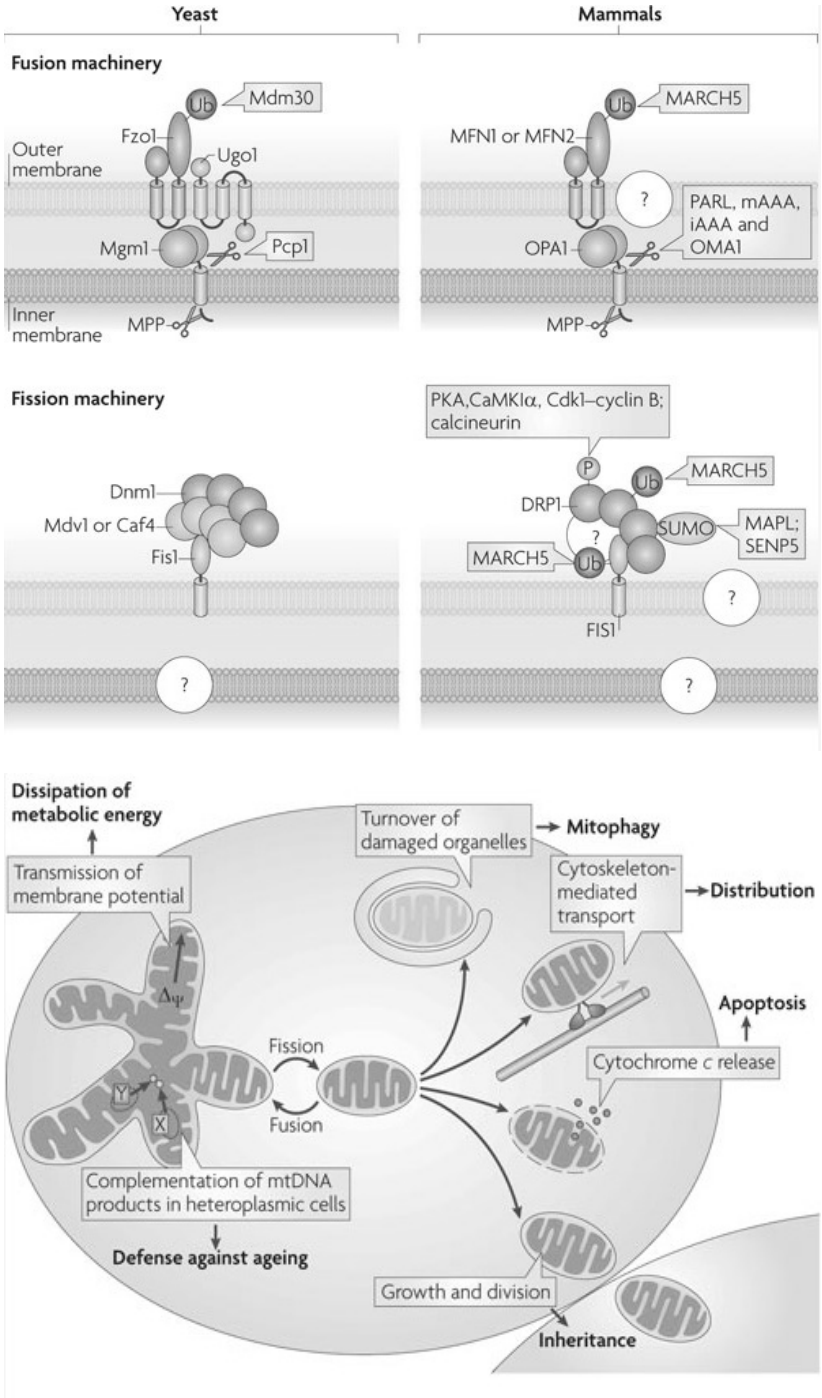


Figure 1.4. Biological functions of mitochondrial dynamics. Mitochondrial fission and fusion are crucial to maintain functional mitochondria upon metabolic or environmental stresses. Fusion attenuates mitochondrial stress by mixing the contents of partially damaged or depolarized mitochondria by complementation. Fission allows the constitution of new mitochondria and contributes to quality control and turnover (together with mitophagy) by enabling the clearance of damaged mitochondria that may induce apoptosis during high cellular stress. The disruption in the homeostasis of these processes affect normal development, and they have been implicated in human diseases. The Figure was adapted from Westermann B, 2010.

Similar to fusion, mitochondrial fission also plays a key role in cell death and aging (Westermann, 2010). As mitochondria propagate by expansion and division of pre-existing organelles, mitochondrial inheritance depends on mitochondrial fission (Figure 1.4). Furthermore, mitochondrial division is important for several developmental and cell differentiation processes, including embryonic development in *C. elegans* and formation of synapses and dendritic spines (Seo *et al.*, 2010).

Additionally, the mitochondrial fission machinery actively participates in apoptosis by inducing fragmentation of the mitochondrial network prior to cytochrome *c* release and caspase activation (Westermann, 2010, Westermann, 2012) (Figure 1.4). In fact, excessive mitochondrial fission appears to be a requisite step in apoptotic pathways, at least for the normal rate of cytochrome *c* release and caspase activation, as several components of the mitochondrial fission machinery (DRP1 and FIS1) have been implicated in programmed cell death progression (Frank *et al.*, 2001, Seo *et al.*, 2010, Westermann, 2010). Dnm1p, the yeast orthologue of DRP1, has been shown to promote mitochondrial fission and cell death following exposure to environmental stress. Increased fission events are also associated with decreased complementation and mitochondrial function, loss of mtDNA integrity and increased oxidative damage, eventually leading to cell death (Seo *et al.*, 2010, Westermann, 2010).

In yeast, mitochondrial fission is mainly dictated by the activity of four proteins, including Fis1p in the outer membrane and three cytosolic proteins (Dnm1p, Mdv1p, and Caf4p) that assemble at the organellar surface near the assembly site (Westermann, 2008, Westermann, 2010) (Figure 1.4). Yeast cells defective in outer membrane fission contain complex, extensively interconnected mitochondrial nets due to ongoing fusion unrestrained by fission. Mostly from yeast studies, the mitochondrial fission process has been characterized to some detail. Dnm1p is a dynamin-related GTPase that assembles into an oligomeric spiral-like structure on the mitochondrial outer membrane (Ingerman *et al.*, 2005). Assembly of this OM complex is mediated by Fis1p and Mdv1p, the latter acting as an adaptor between Fis1p and Dnm1p-GTP domain (Westermann, 2010). Ultimately, these oligomeric structures collapse and separate into two newly separated mitochondria (Ingerman *et al.*, 2005).

1.3.3.2.2. Autophagy and mitophagy in cellular damage control

I. Autophagy

Autophagy is an evolutionarily conserved process in eukaryotic cells that involves the sequestration of cytoplasmic cargo into double-membrane vesicles called autophagosomes. After their formation, autophagosomes fuse to the vacuole (lysosome in mammals), within which the inner membrane and the cargo are degraded (Loewith & Hall, 2011, Ryter *et al.*, 2013, Russell *et al.*, 2014) (Figure 1.5). The importance of autophagy and autophagic-like processes comes from their recognition as key regulators in normal development and cellular homeostasis whose dysfunction with aging is associated with the onset of diseases including cancer, diabetes, neurodegeneration, cardiovascular and hepatic ailments (Boya *et al.*, 2013).

A basal level of autophagy is crucial for routine clearance of cytoplasmic components under normal conditions. It is also critical for protein and organelle homeostasis and turnover and cellular quality control in post-mitotic differentiated cells, such as neurons (Mizushima & Levine, 2010). In addition, autophagy becomes activated in response to starvation, therefore providing a source of nutrients and energy to sustain overall cellular energetics and metabolism (Mizushima & Levine, 2010, Loewith & Hall, 2011, Laplante & Sabatini, 2012). Autophagy is also triggered as an adaptive response to a broad range of other extracellular or intracellular stimuli, namely hypoxia, heat stress, ROS and accumulation of damaged cytoplasmic components (Levine & Klionsky, 2004). Three major subtypes of the autophagy have been described: the non-selective macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Ravikumar *et al.*, 2010, Yang & Klionsky, 2010).

Macroautophagy is the most prevalent form of autophagy and will be herein referred to as autophagy. It is the process by which biomolecules or entire organelles are engulfed in autophagosomes and subsequently degraded by vacuolar/lysosomal hydrolases in a non-selective manner (Russell *et al.*, 2014). Autophagy plays many roles in the cell, namely in starvation adaptation and general metabolism as well as development and differentiation (Ravikumar *et al.*, 2010, Yang & Klionsky, 2010, Ryter *et al.*, 2013). Microautophagy is a process in

which the cytoplasm is directly engulfed at the surface of the degradative organelle (the vacuole or lysosome) without the production of autophagosomes. The membrane invaginates and pinches off to form an internal autophagic vesicle containing cytoplasmic material (Wang & Klionsky, 2003). Both macroautophagy and microautophagy are essentially non-selective, in the way that autophagosomes and vacuole invaginations do not appear to discriminate the sequestered material. The selective autophagy of particular organelles has been described, namely mitophagy, pexophagy and ribophagy, which are involved in the selective turnover of mitochondria, peroxisomes and ribosomes, respectively. Although non-selective autophagy plays an essential role in survival by nitrogen starvation, by providing amino acids to the cell, selective autophagy is more likely to have a function in the maintenance of cellular structures, both under normal conditions as a “housecleaning” process, and under stress conditions by eliminating altered organelles and macromolecular structures.

The chaperone-mediated autophagy (CMA) is a selective form of autophagy, so far only detected in mammalian cells, that is activated during long-term nutrient deprivation and proteostasis (Cuervo & Wong, 2014). CMA does not involve the formation of a double membrane vesicle and targets chaperones to proteins that contain a motif biochemically related to the pentapeptide KFERQ. The chaperone-KFERQ-containing protein complex then binds LAMP-2A (lysosome-associated membrane protein) receptors on the lysosome membrane and translocates the target protein into the lysosomes for degradation (Cuervo & Wong, 2014).

Autophagy can also be involved in biosynthetic-related processes. The cytoplasm to vacuole (Cvt) targeting pathway is a yeast-specific autophagy-like pathway in which vacuolar hydrolases, such as aminopeptidase I (ApeI) and α-mannosidase, are selectively delivered to the vacuole and converted into enzymatically active forms at the vacuolar lumen. This pathway employs most of the autophagic machinery (Scott *et al.*, 1996, Reggiori & Klionsky, 2013).

Thirty-five autophagy-related genes (ATG) in yeast have been so far identified, and, many of them were essential to identify homologues in higher eukaryotes. ATG proteins are organized in functional complexes that mediate the diverse steps of macroautophagy and other selective forms of autophagy: induction/initiation, vesicle nucleation, cargo recognition and packaging, vesicle

expansion and sealing, fusion with the lysosome (vacuole in yeast), vesicle breakdown and recycling of the resulting macromolecules (Maiuri *et al.*, 2007) (Figure 1.5).

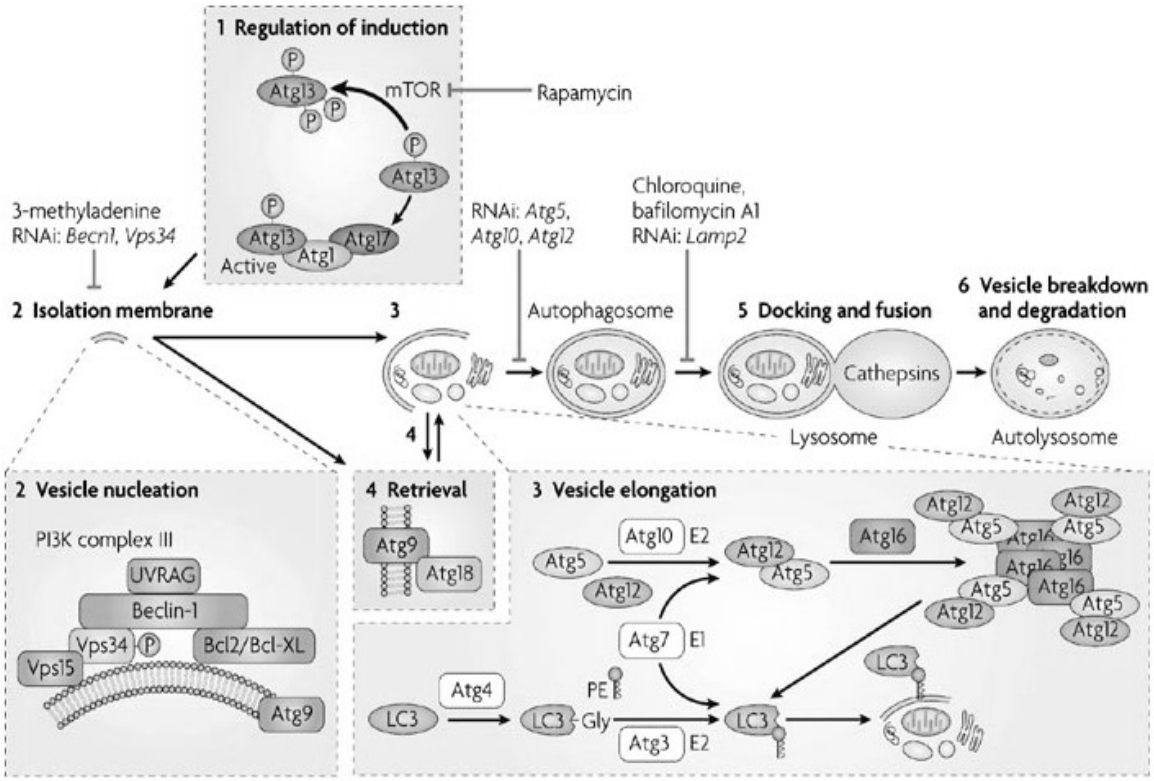


Figure 1.5. Schematic diagram of the various stages of autophagy. Autophagy induction upon mTOR inhibition, a sensor of nutrients (aminoacids), allows the activation of the ULK1 kinase complex. In nucleation, the Class III PI(3)K complex forms PI(3)P, which is necessary for formation of the isolation membrane. The membrane expands to engulf cytosolic contents in vesicle elongation, a process that requires the two ubiquitin-like conjugation steps of ATG5–ATG12 and LC3/At8p–PE. In vesicle retrieval, the transport of ATG9 between the PAS and non-PAS sites is necessary for autophagosome formation and requires ATG18. During vesicle maturation, trafficking and fusion of the fully enclosed double-membrane autophagosome to various endosomal compartments occurs, eventually fusing to the lysosome (vacuole in yeast) to form the autolysosome. In the final stage, degradation of the contents of the autolysosome occurs by the resident lysosomal enzymes. Although the process is described for mammalian cells, similar features are also observed for yeast cells. The Figure was obtained from Maiuri *et al.*, 2007.

II. Autophagy regulation and cell signalling pathways

In yeast, some signalling pathways have been characterized as playing an important role in the regulation of autophagy. The regulatory proteins of these pathways are TORC1, Sch9p, PKA, and Pho85p protein kinases (Reggiori & Klionsky, 2013).

Under nutrient-rich conditions, autophagy is inhibited because TORC1 is active and hyperphosphorylates Atg13p (Kamada *et al.*, 2010, Reggiori & Klionsky, 2013), resulting in a lower affinity for the serine-threonine protein kinase Atg1p and Atg17p to induce the process (Figure 1.5). PKA and Sch9p are also involved in the regulation of Atg13p phosphorylation and localization to the phagophore assembly site (PAS) (Stephan *et al.*, 2009), although the mechanisms involved are yet fully understood.

When TORC1 activity is inhibited, either by rapamycin or starvation, Atg13p is rapidly dephosphorylated by unknown mechanisms (to yield a hypo-phosphorylated form of Atg13p) and interacts with Atg1p (Kamada *et al.*, 2010, Reggiori & Klionsky, 2013). The Atg1p-Atg13p protein complex then associates with Atg17p, which is part of a ternary complex with Atg29p and Atg31p. The Atg1p-Atg13p protein complex then recruits other Atg proteins to the phagophore assembly site and controls their assembly and dynamics (Reggiori & Klionsky, 2013). Autophagosome nucleation requires a complex containing Atg9p and the class III phosphatidylinositol 3-kinase Vps34p, the latter generating phosphatidylinositol 3-phosphate (Maiuri *et al.*, 2007, Reggiori & Klionsky, 2013) (Figure 1.5).

The expansion of autophagosomal membranes involves two ubiquitin-like proteins, Atg12p and Atg8p, an E1 ubiquitin activating enzyme (Atg7p), two analogues of ubiquitin-conjugated enzymes (Atg10p and Atg3p), an Atg8p modifying protease (Atg4p), the protein target of Atg12p attachment (Atg5p) and Atg16p (Figure 1.5). In the first ubiquitination reaction, the E1-like Atg7p and the E2-like Atg10p promote the association of Atg12p with Atg5p (Suzuki *et al.*, 2001, Suzuki *et al.*, 2007). This conjugate subsequently interacts with Atg16p to generate pre-autophagosomal structures (Mizushima *et al.*, 1999). In the second ubiquitin reaction, Atg8p is cleaved by the protease Atg4p and conjugated to phosphatidylethanolamine (PE) by Atg7p (E1-like) and Atg3p (Kim *et al.*, 1999,

Kirisako *et al.*, 1999). This lipidated form of Atg8p is essential to drive proper autophagosome biogenesis (Figure 1.5). Upon completion of autophagosome formation, the Atg12p–Atg5p–Atg16p protein complex is released into the cytosol, whereas Atg8p-PE remains stably associated with the autophagosomal membranes (Kirisako *et al.*, 2000). At this point, the outer autophagosomal membrane fuses with the vacuolar membrane to produce an autophagic body (autolysosome in mammalian cells) (Reggiori & Klionsky, 2013) (Figure 1.5). Several proteins are known to regulate the autophagosome-to-vacuole fusion step, namely the Rab protein Ypt7p (Kim *et al.*, 1999), its GDP-exchange factor Ccz1p-Mon1p (Wang *et al.*, 2003), and several SNARE proteins such as Vam3p, Vam7p, Vti1p and Ykt6p along with the class C Vps/HOPS complex, which are essential for proper tethering and fusion (Reggiori & Klionsky, 2013). The remaining single-membrane that envelops the cargo is lysed and the population of Atg8-PE together with the enclosed cargo are released into the vacuolar lumen and degraded by resident vacuolar hydrolases (proteases, lipases, nucleases and glucosidases). The resulting degradation products are released back into the cytosol through the activity of specific membrane permeases for recycling (Reggiori & Klionsky, 2013).

III. Autophagy and lifespan

The relationship between CLS and autophagy is extremely complex and not fully understood. Autophagy appears to be a common downstream process of multiple cellular pathways with well-known roles in longevity regulation and disease (Madeo *et al.*, 2010). The upregulation of autophagy extends chronological lifespan in mice, C. *elegans*, yeast and other organisms. Importantly, the TORC1-Sch9p and the Ras/cAMP-dependent protein kinase proteins, which integrate the network of nutrient- and energy-sensing pathways that regulate autophagy, are known to be involved in proper regulation of longevity pathways (Wei *et al.*, 2008, Galdieri *et al.*, 2010, Evans *et al.*, 2011, Swinnen *et al.*, 2013). Recently, Hansen *et al.* found that dietary restriction and TOR inhibition in *C. elegans* produce an "autophagy" phenotype and that inhibiting genes required for autophagy prevents dietary restriction and TOR inhibition from extending lifespan, corroborating with this conception (Hansen *et al.*, 2008).

Screenings performed in yeast have demonstrated that mutants for genes encoding proteins of the autophagic machinery (*ATG5*, *ATG7*, *ATG8*, *ATG12* and *ATG18*) are short-lived (Matecic *et al.*, 2010) and some genes are required to extend CLS upon rapamycin treatment (*ATG1* and *ATG7*) (Alvers *et al.*, 2009). Suppression of autophagy by knockdown of essential autophagy genes triggers apoptosis or necrosis in cells that would otherwise survive under stress conditions (Kourtis & Tavernarakis, 2009, Suzuki *et al.*, 2011). Autophagy appears to serve primarily a cytoprotective function by maintaining nutrient and energy homeostasis during starvation or by degrading damaged cellular components and invasive pathogens (Reggiori & Klionsky, 2013). Although autophagy is a predominantly pro-survival mechanism, it can also play a role in cell death, which is not restricted to developmental programmed cell death, but extends to cell death that occurs in many pathological conditions (Codogno & Meijer, 2005, Tsujimoto & Shimizu, 2005). Excessive autophagy induced by extreme conditions such as toxins and necrosis-triggering insults might cause uncontrollable degradation or sequestration of cells contents resulting in undesirable cell death if not properly regulated (Codogno & Meijer, 2005).

IV. Mitophagy

The mitochondrial theory of aging predicts that an accumulation of oxidative damage and mtDNA mutations is associated with the onset of age-associated pathologies and cell death (Shokolenko *et al.*, 2014). Mitophagy promotes the removal of damaged/dysfunctional or oxidized mitochondria and contributes to the homeostatic maintenance of sustainable mitochondrial function and thereby allows an efficient process for ATP production and maintenance of proper cellular energetics (Seo *et al.*, 2010).

There is some evidence in yeast studies suggesting that damaged mitochondria are eliminated by mitophagy. For example, interference with F_1F_o-ATPase biogenesis in a temperature sensitive *fmc1*^{ts} mutant (Priault *et al.*, 2005) or osmotic swelling of mitochondria caused by depletion of the mitochondrial K^+/H^+ exchanger Mdm38p induce mitophagy (Nowikovsky *et al.*, 2007). It is conceivable to assume that mitophagy also mitigates the deleterious effect of mtDNA mutations in heteroplasmic cells, together with mitochondrial dynamics (Figure

1.4). Supporting this view, Mao *et al.* have recently reinforced the important link between mitophagy and mitochondrial dynamics (Mao *et al.*, 2013). On this basis, both processes seem to act in a coordinate manner to assure the proper connectivity of the mitochondrial network, which is an important factor that determines cell's fate (Figure 1.4).

The core autophagic machinery used is common with other types of autophagy. The requirement of several ATG genes for mitophagy has been reported by several groups and the screening of ATG mutants in *S. cerevisiae* allowed the identification of some ATG proteins selectively involved in mitophagy, namely Atg32p and Atg33p (Kanki *et al.*, 2009a, Kanki & Klionsky, 2010a). Recently, the Slt2p and Hog1p mitogen activated protein kinases (MAPK) were identified as key mediators of mitophagy by regulating the phosphorylation of Ser114 and Ser119 on Atg32p (Aoki *et al.*, 2011, Mao *et al.*, 2011) (Figure 1.6).

Importantly, Slt2p is required for proper recruitment of mitochondria to PAS (Mao *et al.*, 2011). Whi2p, Uth1p, and Aup1p are also required for mitophagy, although the function of these proteins in mitophagy remains to be established (Bhatia-Kiššová & Camougrand, 2010, Hirota *et al.*, 2012) (Figure 1.6). Casein kinase 2 (CK2) phosphorylates Atg32p and activates mitophagy in yeast, but the relevance for the process is not yet understood (Kanki *et al.*, 2013). In an elegant work, Bockler and Westermann identified a complex connecting mitochondria and the ER structure termed ERMES (ER mitochondria encounter structure), which mediated the mitochondrial-ER tethering (mitochondrial-ER contacts) and is essential for the formation of the isolation membrane destined to the formation of mitophagosomes (Bockler & Westermann, 2014).

In yeast studies, there are several ways to induced mitophagy. The most common is the incubation in non-fermentable medium to induce the proliferation of mitochondria (Kissova *et al.*, 2004, Kanki *et al.*, 2009b) (lactate or glycerol as sole carbon sources). Cells can then be grown upon nitrogen starvation conditions to induce mitophagy (Mao *et al.*, 2011). Rapamycin has also been reported to induce the process (Mendl *et al.*, 2011). The importance of mitophagy is demonstrated by its association with the onset of age-associated ailments, such as neurodegenerative diseases (Mijaljica *et al.*, 2010).

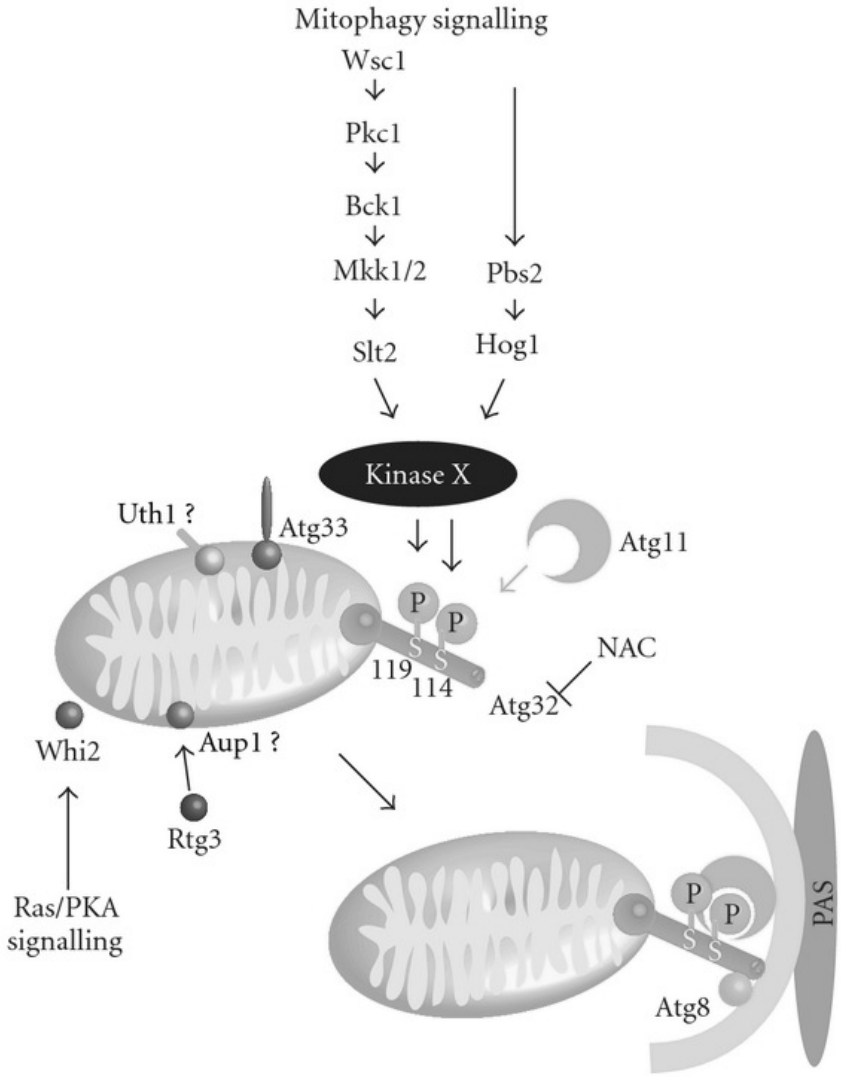


Figure 1.6. Mitophagy associated signalling in yeast. The MAPK Slt2p and Hog1p regulate the phosphorylation of Atg32p at s Ser114 and Ser119 residues. This phosphorylation is important for the Atg11p-Atg32p interaction. Atg11p recruits mitochondria to the PAS to be enclosed in mitophagosomes. Proteins Atg33p, Whi2p, Uth1p, and Aup1p have been reported to be important for the induction of mitophagy. However, their regulatory role remains to be established. The Figure was modified from Hirota *et al*., 2012.

1.4. Sphingolipids

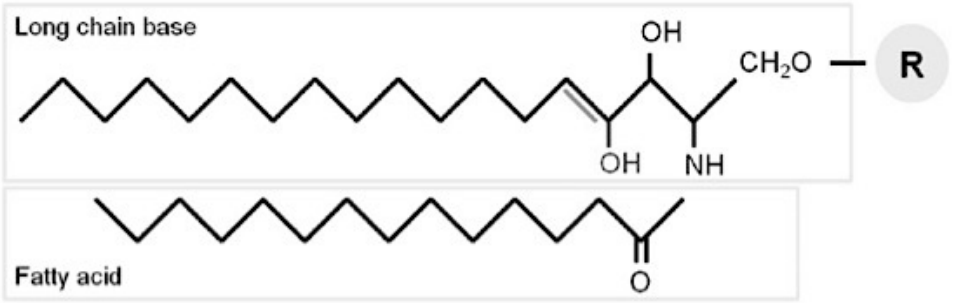
1.4.1. Structure

Sphingolipids are important structural components of cell membranes found in essentially all animals, plants and fungi, as well as some prokaryotic organisms (Hannun & Obeid, 2008). They are mostly found on the outer leaflet of the plasma membrane, although they are also present at membranes of different organelles at variable ratio (Hannun & Obeid, 2008). Furthermore, they are major constituents of lipoproteins. Sphingolipids, including sphingosine, ceramide and sphingosine-1-phosphate (S1P), have emerged as core metabolites in this metabolism as they regulate a vast number of cellular processes including cell growth, adhesion, migration, senescence, apoptosis, endocytosis, and most recently, autophagy in yeast and higher eukaryotes (Dickson, 2008, Hannun & Obeid, 2008, Young *et al.*, 2013). Since they have the ability to modulate the activity of some proteins and the activation of some signalling pathways, variations in the relative levels of different sphingolipid species result in important changes in overall cellular functions and fate (Hannun & Obeid, 2008).

From a structural point of view, sphingolipids have an amphipathic nature and are composed by a long chain sphingoid base (LCB) (sphingosine (SPH) in mammals, and phytosphingosine (PHS) and dihydrosphingosine (DHS) in yeast), with the 2-amino group amide-linked to a fatty acid, thereby forming the core unit, to which polar groups are added at C1-hydroxyl group to give rise to different types of sphingolipids (Hannun & Obeid, 2008, Rego *et al.*, 2014) (Figure 1.7). The nature of the fatty acid (carbon length, degree of unsaturation and hydroxylation) along with other modifications of the long-chain bases and the polar head group define the vast family of sphingolipids.

1.4.2. Bioactive sphingolipids and aging

Sphingolipid metabolism is highly complex and interconnected, which enable cells to orchestrate different cellular responses by regulating sphingolipid interconversions (Hannun & Obeid, 2008, Rao *et al.*, 2013).



Yeast substituent R	Sphingolipid
Hydrogen	Dihydroceramide or phytoceramide
Inositol phosphate	Inositol phosphorylceramide
Mannose - inositol phosphate	Mannosylinositol phosphorylceramide
Inositol phosphate – mannose – inositol phosphate	Mannosyldiinositol phosphorylceramide

Figure 1.7. The general structure of sphingolipids. Sphingolipid are composed of LCBs, which can be linked to a fatty acid via an amide bond and to a polar head of different chemistry. The nature of the fatty acid and the polar headgroup define the extended family of sphingolipids. The Figure was modified from Rego *et al*., 2014.

In response to both extracellular stimuli (UV, hypoxia, toxins, heat stress, etc.) and alterations in cellular physiology, the enzymes involved in sphingolipid metabolism act in a coordinated manner to regulate not only the levels of individual bioactive lipids, but also their metabolic interconversion and the physiological response generated after proper integration of cellular signalling (Hannun & Obeid, 2008).

The sphingolipids ceramide, sphingosine and sphingosine-1-phosphate are the main representatives of sphingolipid metabolism and play crucial roles in the regulation of many cellular processes (Hannun & Obeid, 2008, Rego *et al.*, 2014). The first sphingolipid to be identified was sphingosine and it exerts pleiotropic effects on protein kinases and other targets (Hannun & Obeid, 2008). Sphingosine and its related sphingoid bases have roles in regulating the actin cytoskeleton, endocytosis, cell cycle and apoptosis. Ceramide mediates many cell-stress responses that include the promotion of pro-apoptotic events and cell senescence, by modulating the activity of ceramide-activated protein kinases (e.g. PKCζ) and phosphatases [ceramide-activated protein phosphatase (CAPP), phosphoprotein 1 (PP1) and phosphoprotein 2A (PP2A)] (Hannun & Obeid, 2008, Rao *et al.*, 2013) (Figure 1.8). On the other hand, S1P promotes cell proliferation and survival by

acting in an autocrine manner on S1P receptors (Hannun & Obeid, 2008, Rao *et al.*, 2013) (Figure 1.8). Consequently, alterations in the relative amounts of sphingosine-1-phosphate and sphingosine/ceramide have significant effects on cell physiology and metabolism and ultimately on cell fate (Figure 1.8). This explains why sphongolipid metabolism is highly regulated (Hannun & Obeid, 2008, Rao *et al.*, 2013). Other components of the family of sphingolipids include ceramide-1-phosphate (C1P), involved in inflammation and vesicular trafficking, glucosylceramide, mostly associated with post-Golgi trafficking and drug resistance, lyso-sphingomyelin and dihydroceramide (Hannun & Obeid, 2008, Rao *et al.*, 2013).

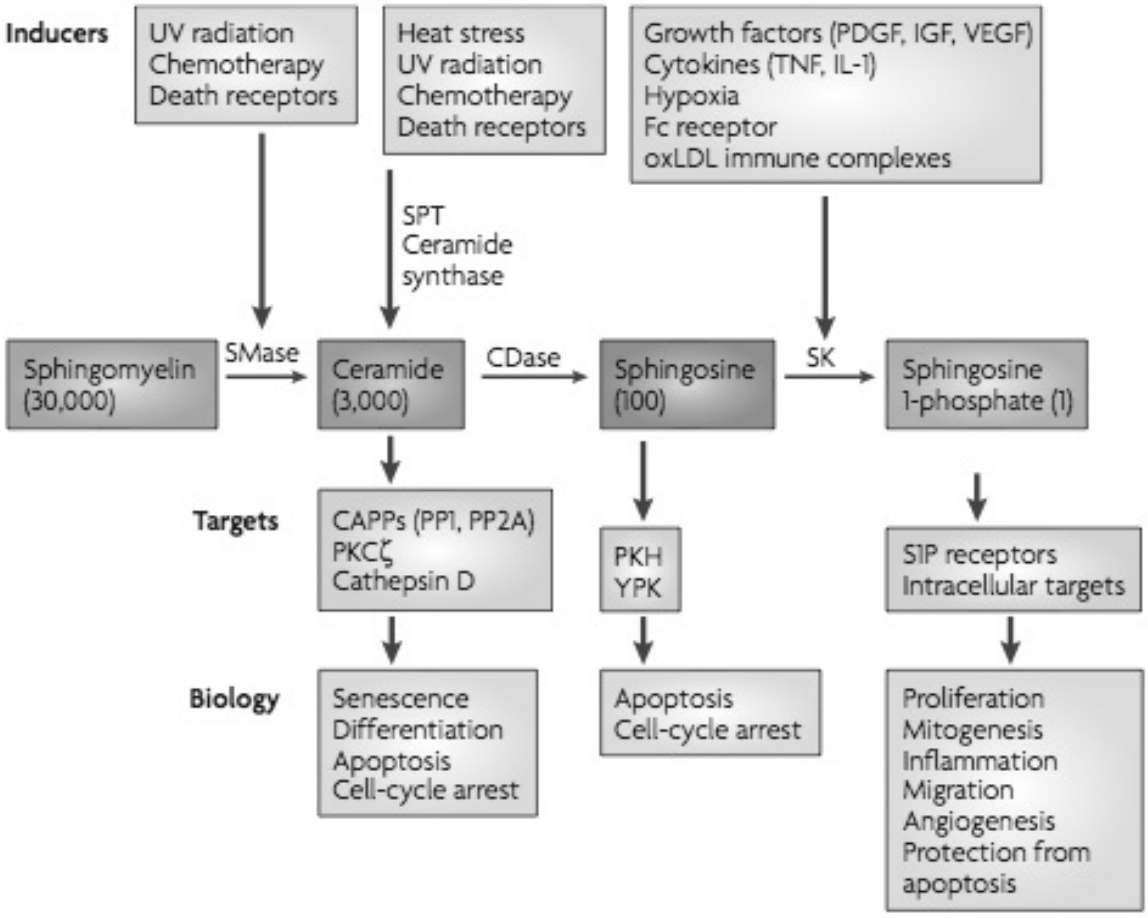


Figure 1.8. **Sphingolipid metabolism and signalling**. In response to several stimuli, sphingolipid metabolism can be adaptively activated. Whereas ceramide and sphingosine are mostly associated with apoptosis and cellular senescence by activating protein kinases and phosphatases, sphingosine-1-phosphate (S1P), conversely, promotes cell proliferation and protection from apoptosis by acting on receptors, usually in an autocrine manner. Therefore, the regulation of ceramide/S1P is crucial to determine cell fate. The Figure was adapted from Hannun & Obeid, 2008.

The importance of sphingolipid signalling derives from the early recognition of their contribution in the pathobiology of human cancers and other human ailments such as diabetes and heart disease, microbial infections, neurological and immune dysfunctions (Kolter & Sandhoff, 2006, Ozbayraktar & Ulgen, 2009, Kolter, 2011, Hla & Dannenberg, 2012, Young *et al.*, 2013). Additionally, sphingolipids have been implicated in the regulation of stress responses and longevity. In mammals, ceramide levels and SMase activity increase and induce a senescent phenotype morphologically and biochemically (inhibition of cell cycle progression, inhibition of the PKC/PLD pathway, induction of Rb dephosphorylation and inhibition of the pro-proliferative AP1 activity) (Obeid *et al.*, 1993, Obeid & Venable, 1997, Hannun & Luberto, 2000). Senescence and growth inhibition were also correlated with the inhibition of telomerase transcription and activity by ceramide (Ogretmen *et al.*, 2001). Yeast mutants lacking Ydc1p (dihydroceramidase) are characterized by increased CLS whereas the overexpression of *YDC1* triggers mitochondria and vacuolar fragmentation, apoptosis and accelerated aging in yeast (Aerts *et al.*, 2008). Genes involved in sphingolipid metabolism (*LAG1*, *YPC1*, *YSR3*, *IPT1*, and *LCB5*) show variable expression in senescent and apoptotic cells (Laun *et al.*, 2005). More recently, it was shown that the downregulation of sphingolipid biosynthesis increases yeast CLS in part due to a reduction in long-chain bases (LCBs) mediated activation of the Pkh1p-Sch9p pathway (Huang *et al.*, 2012). Furthermore, ceramide synthase (Lag1p) and LCB kinase (Lcb4p) activities decrease upon entry into the stationary phase, leading to a large increase in the levels of LCBs (Lester *et al.*, 2013).

Over the past few decades, many studies attempted to unveil the role of sphingolipids in signal transduction pathways involved in the regulation of these biological processes. However, the mechanisms by which sphingolipids control many aspects of cell physiology and metabolism remains to be characterized in more detail.

1.4.3. Yeast sphingolipid metabolism

The sphingolipid metabolism is highly conserved between yeast and mammals and shares a similar spatial organization (Figure 1.9).

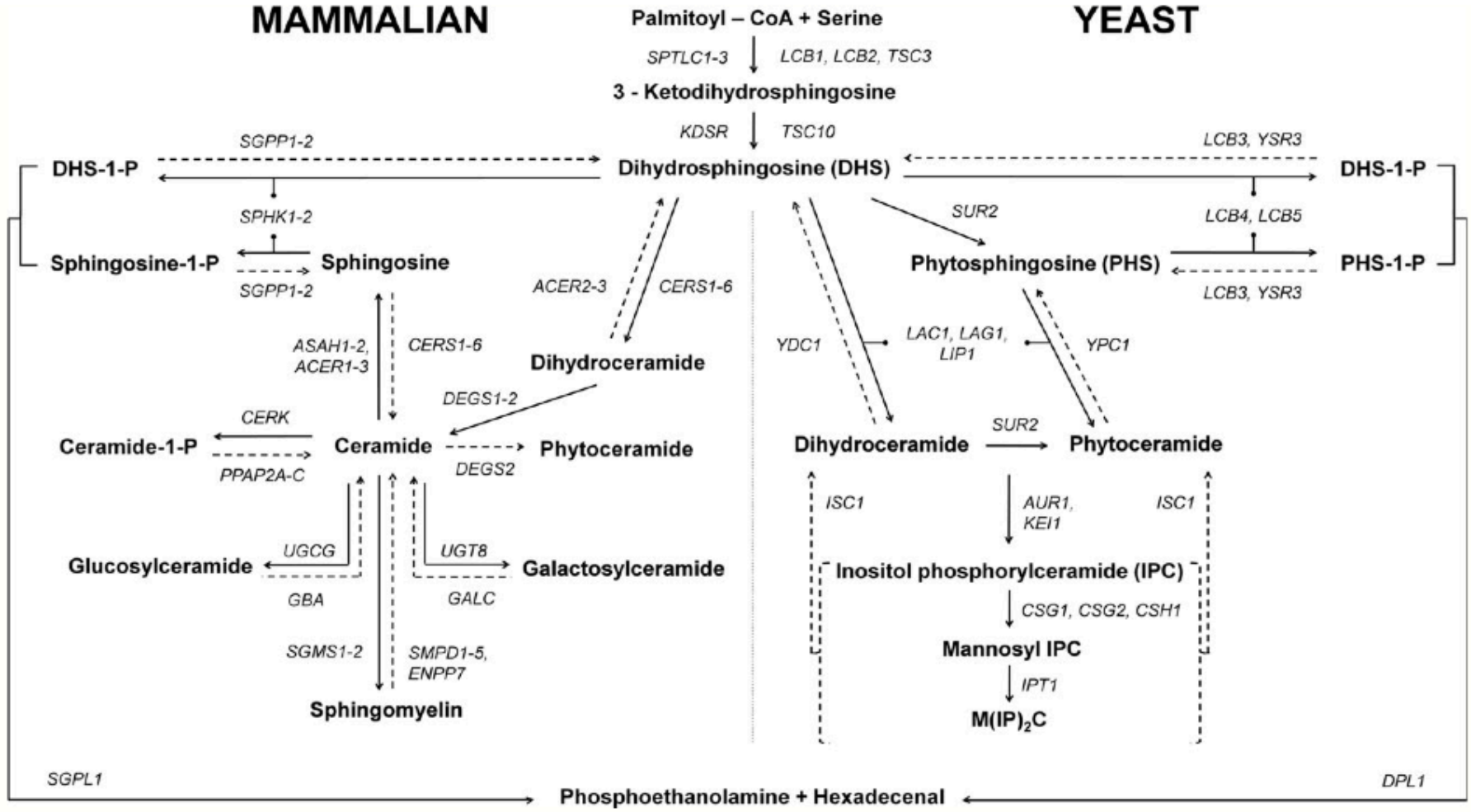


Figure 1.9. Schematic representation of the highly conserved sphingolipid metabolism. Interconversions and the enzymes involved in sphingolipid metabolism are shown. The Figure was obtained from Rego *et al*., 2014.

It takes place in the ER and the subsequent processes occur in the Golgi apparatus, where the delivery of intermediate metabolites and synthesis of complex sphingolipids manifest (Hannun & Obeid, 2008, Rego *et al.*, 2014). Sphingolipids metabolism involves the biosynthesis and recycling of a large of number of intermediate metabolites. Despite the diversity of structure and function of sphingolipids, this metabolism is governed by an integrated network of common synthetic and catabolic pathways in response to different stimuli (Hannun & Obeid, 2008, Rego *et al.*, 2014) (Figure 1.9).

1.4.3.1. *De novo* biosynthesis in the ER

The first and rate-limiting step in yeast sphingolipid metabolism involves the condensation of serine and palmitoyl-CoA in the ER in a reaction catalyzed by serine palmitoyltransferase (SPT), yielding 3-keto-dihydrosphingosine (Dickson & Lester, 1999). This first step is the only known entry route in the biosynthetic sphingolipid metabolism and the subtract availability in this reaction regulates the flux through the pathway (Alvarez-Vasquez *et al.*, 2005). SPT is encoded by two homologous subunits, *LCB1* and *LCB2*, both of which are required for proper activity (Nagiec *et al.*, 1994). In yeast, a third small subunit of SPT, Tsc3p (temperature-sensitive suppressor of calcium sensitivity) is required for the activity by forming a heterodimer with Lcb1p and Lcb2p (Gable *et al.*, 2000). Tsc3p function is unknown but it influences Lcb2p activity in the Tsc3p-Lcb2p-Lcb1p complex (Monaghan *et al.*, 2002).

More recently, some important regulators of the sphingolipid biosynthetic pathway have been uncovered. An important regulator of SPT activity is the TORC2-Pkh1p-Ypk1p-Orm1/2p pathway (Berchtold *et al.*, 2012, Niles & Powers, 2012, Muir *et al.*, 2014). In response to mechanical changes or depletion in the content of complex sphingolipids at the plasma membrane (e.g. upon treatment with myriocin and aureobasidin A), TORC2 becomes activated and stimulates the *de novo* biosynthesis of sphingolipids via phosphorylation and activation of Ypk1p at the hydrophobic motif (HM) site (Roelants *et al.*, 2011, Berchtold *et al.*, 2012, Niles & Powers, 2012) (Figure 1.10). Apart from TORC2, the Pkh1/2p protein kinases also phosphorylate Ypk1p at the activation loop (A-loop) in the plasma membrane, which is required for full activation of Ypk1p (Berchtold *et al.*, 2012)

(Figure 1.10). After being activated, Ypk1p phosphorylates and inactivates Orm1/2p, two evolutionarily conserved integral membrane proteins of the ER (Breslow *et al.*, 2010, Roelants *et al.*, 2011) (Figure 1.10). Since Orm1/2p are inhibitors of SPT, (Breslow *et al.*, 2010), which catalyzes the first step in sphingolipid biosynthesis, their inactivation by a Ypk1p-dependent phosphorylation alleviates SPT inhibition and boosts sphingolipid production (Breslow *et al.*, 2010, Roelants *et al.*, 2011, Berchtold *et al.*, 2012) (Figure 1.10). In this regulatory network, the pleckstrin homology (PH) domain proteins Slm1p/Slm2p are recruited to the plasma membrane by TORC2-mediated phosphorylation and play an essential role in the activity of Ypk1/2p by increasing the efficiency of phosphorylation of Ypk1p by TORC2 and Pkh1/2p (Tabuchi *et al.*, 2006, Berchtold *et al.*, 2012, Niles & Powers, 2012) (Figure 1.10).

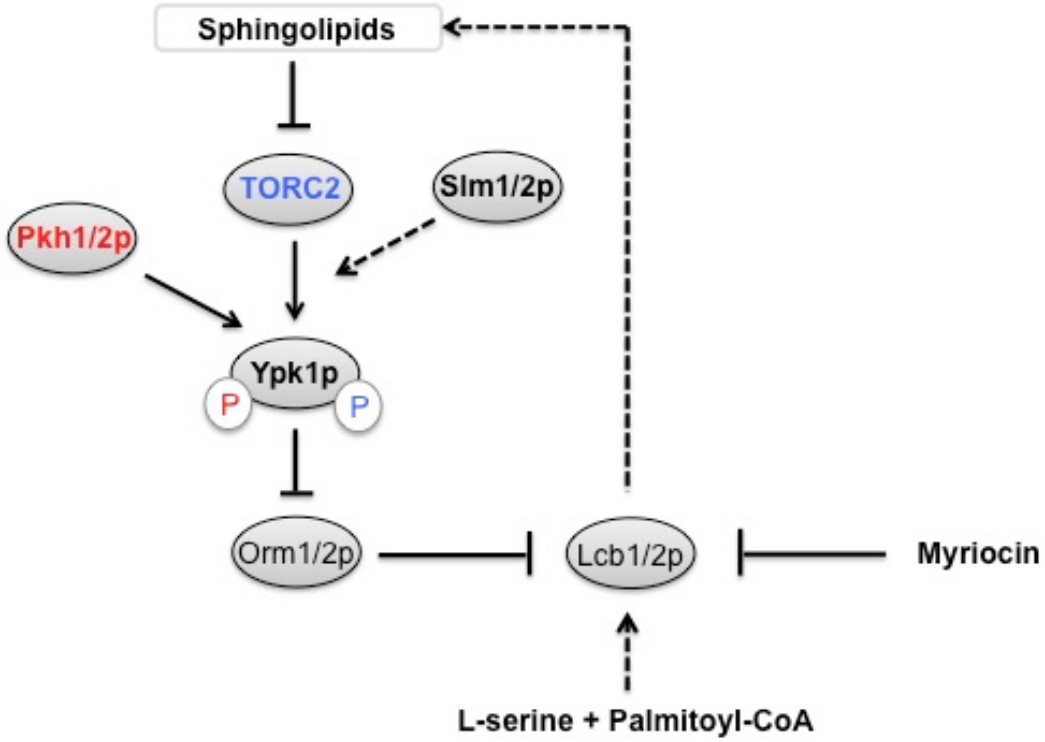


Figure 1.10. TOR Complex 2-Pkh1/2p-Ypk1p-Orm signalling maintains sphingolipid homeostasis by targeting the *de novo* biosynthesis pathway. In response to sphingolipid depletion (e.g. myriocin treatment), TORC2 is activated and phosphorylates the downstream effector Ypk1p, which is also phosphorylated by Pkh1/2p for full activation. Once Ypk1p is activated, it phosphorylates and inhibits the Orm1/2p proteins, which act as inhibitors of SPT (Lcb1/2p protein complex). As a result, Orm1/2p-mediated inhibition of SPT activity is alleviated, therefore boosting sphingolipid biosynthesis.

It is noteworthy that TORC2 also stimulates ceramide synthase through Ypk2p (Aronova *et al.*, 2008) (a paralog of Ypk1p) and also inhibits Isc1p, the homologue of mammalian neutral sphingolmyelinase 2 (nMase2), via modulation of Slm1/2p (Tabuchi *et al.*, 2006). Thus, Slm1p and Slm2p regulate both ceramide biosynthesis, via the TORC2-Ypk1/2p-SPT-ceramide synthase cascade, and ceramide turnover by hydrolysis of complex sphingolipids mediated by Isc1p.

After 3-keto-dihydrosphingosine generation, this intermediate is converted to the LCB dihydrosphingosine (DHS) in an NADPH-dependent manner by Tsc10p protein (Figure 1.9). Sur2p/Syr2p catalyzes the hydroxylation of DHS at C4 position to produce phytosphingosine (PHS) (Haak *et al.*, 1997) (Figure 1.9). Structurally, DHS and PHS also vary in the chain length: DHS contains 16, 18 or 20 carbons while PHS presents 18 or 20 carbons (Dickson, 2008). Together, DHS and PHS constitute the LCBs in yeast, and both can undergo either phosphorylation at C1 or N-acylation. DHS and PHS can be phosphorylated by two LCB kinases, encoded by *LCB4* and *LCB5* genes, forming DHS-1-phosphate and PHS-1-phosphate, respectively (Nagiec *et al.*, 1998) (Figure 1.9). Finally, these phosphorylated products can be either dephosphorylated back to DHS and PHS by the phosphatases Lcb3p/Ysr2p and Ysr3p or catabolized by dihydrosphingosine-1-phosphate lyase (Dpl1p) (Skrzypek *et al.*, 1999) to generate palmitaldehyde and phosphoethanolamine (Figure 1.9). The production of these non-sphingoid molecules constitutes the only known exit route from the sphingolipid metabolism (Hannun & Obeid, 2008).

1.4.3.2. Ceramide generation and breakdown

Apart from phosphorylation, DHS or PHS can be N-acylated to produce the correspondent dihydro- and phytoceramides (Figure 1.9). This reaction requires two ceramide synthases, encoded by *LAG1* (longevity assurance gene 1) and *LAC1* (longevity assurance gene 1 cognate) (Guillas *et al.*, 2001) (Figure 1.9). *LAG1* was firstly associated as a gene involved in the aging process since its expression is diminished in aged yeast cells (D'Mello N *et al.*, 1994). *LAC1* was then identified as a homologue of *LAG1*; both proteins are required for ceramide synthesis (Guillas *et al.*, 2001). These enzymes are highly homologous and present redundant function and the double deletion of *LAG1* and *LAC1* is required

to prevent *de novo* biosynthesis of ceramide (Guillas *et al.*, 2001, Schorling *et al.*, 2001). In addition, Lip1p forms a heteromeric complex with Lac1p and Lag1p and is essential for ceramide synthase activity *in vivo* and *in vitro (Vallee & Riezman, 2005)*. Dihydro- and phytoceramides can be deacylated to form DHS/PHS by ceramidases Ydc1p and Ypc1p (Mao *et al.*, 2000a, Mao *et al.*, 2000b), or can be used as substrates for the biosynthesis of complex sphingolipids (Figure 1.9).

1.4.3.3. Biosynthesis of complex sphingolipids

After ceramide formation, it must be transported from the ER to the Golgi compartment either by a vesicular and non-vesicular transport mechanism involving direct membranous contact between the ER and Golgi compartment (Hannun & Obeid, 2008). Once ceramide reaches the Golgi apparatus, it becomes the substrate for the production of complex sphingolipids, namely inositol-phosphoceramide (IPC), mannosyl-inositol phosphoceramide (MIPC) and mannosyl-di-inositol-phosphoceramide [$M(IP)_2C$] (Figure 1.9).

The first complex sphingolipid, inositol-phosphoceramide (IPC), is formed by transferring a myo-inositol phosphate group from phosphatidylinositol (PI) to ceramide with the concomitant release of diacylglycerol (DAG). This step is catalyzed by the IPC synthase encoded by *AUR1*, an essential gene whose deletion is lethal (Nagiec *et al.*, 1997) (Figure 1.9). The second complex sphingolipid, MIPC, is generated by transferring the mannose group from GDP-mannose onto the inositol 2-OH moiety of IPC (Uemura *et al.*, 2003, Uemura *et al.*, 2007). The enzyme inositol phosphorylceramide mannosyl transferase catalyzes this reaction and can be present in two forms. One contains the Csg1p and Csg2p proteins, and the other contains the Csh1p and Csg2p. The Csg1p and Csh1p appear to be the catalytic subunits, whereas Csg2p performs a regulatory function (Uemura *et al.*, 2007).

The terminal yeast complex sphingolipid made in the Golgi apparatus is $M(IP)_2C$. It is the most abundant complex sphingolipid in yeast and it is synthesized by the addition of another inositol phosphate group to MIPC by a process catalyzed by inositol-phosphotransferase (Ipt1p) (Dickson *et al.*, 1997) (Figure 1.9). Hechtberger *et al.* have reported that complex sphingolipids in *S. cerevisiae* are mostly present in the Golgi compartment, vacuole and plasma

membrane (where they are thought to coexist with ergosterol to form lipid rafts), and also observed in the mitochondria at lower levels, but not in the ER (Hechtberger *et al.*, 1994).

1.4.3.4. Sphingolipid catabolism

I. The Inositol phosphosphingolipid phospholipase C

Complex sphingolipids turnover to regenerate ceramide is performed by inositol phosphosphingolipid phospholipase C (Isc1p), which has phospholipase-C type activity and catalyzes the removal of the polar head groups from complex sphingolipids, releasing dihydroceramide and phytoceramide (Sawai *et al.*, 2000, Rego *et al.*, 2014) (Figure 1.9). Ella *et al*. have firstly demonstrated that yeast presents an enzyme with the ability to hydrolyze mammalian sphingomyelin at neutral pH, despite the absence of this sphingolipid in yeast (Ella *et al.*, 1997). Then, the detection of Isc1p as a membrane-associated phospholipase C-type activity that produced ceramide from IPC, MIPC, and $M(IP)_2C$ was identified with significant homology to bacterial sphingomyelinases (Ella *et al.*, 1997). Consistently, Isc1p overexpression results in an increase of ceramide levels, whereas *ISC1* deletion results in an accumulation of complex sphingolipids (Sawai *et al.*, 2000).

Isc1p belongs to the sphingomyelinase family since it shares very significant homology to bacterial sphingomyelinases of *Bacillus cereus* and *Staphylococcus aureus* (Clarke *et al.*, 2006). In particular, Isc1p is the yeast homolog of mammalian neutral sphingomyelinase type 2 (nSMase2) and shares 30% identity in sequence to its counterpart. From a structural point of view, Isc1p is activated by phosphatidylserine (PS), phosphatidylglycerol (PG), and cardiolipin (CL) at the C-terminus and is dependent on the presence of Mg^{2+} as a cofactor for optimal activity (Sawai *et al.*, 2000, Vaena de Avalos *et al.*, 2004). According to the proposed “tether-and-pull” model, the C-terminus of Isc1p associates with PS, CL, and PG lipids and consequently, the N-terminal catalytic domain is “pulled” to the membrane to interact with the substrates (Okamoto *et al.*, 2003). The activity of Isc1p relies on the highly conserved residues E100, D234, and H334, located at the P-loop-like catalytic domain, which may be involved in Mg^{2+} metal binding,

interaction with the phosphate group, and as a general base in the catalytic reaction, respectively (Okamoto *et al.*, 2003). Ideally, Isc1p presents optimal activity around pH 7.5 and very low activity in acidic and very alkaline conditions (Okamoto *et al.*, 2003).

Interestingly, Isc1p is post-translationally regulated by translocation from the ER into mitochondria upon the transition from fermentative to the respiratory metabolism during the so-called post-diauxic shift (PDS) (Vaena de Avalos *et al.*, 2004). This appears to be associated with the regulation of mitochondrial sphingolipid metabolism and function, namely the generation of α-hydroxylated-phytoceramides, which are necessary for proper function of this organelle (Kitagaki *et al.*, 2007, Kitagaki *et al.*, 2009). The regulatory role of Isc1p on mitochondrial function is reinforced by the fact that Isc1p-deficient cells exhibit a growth defect upon the transition to the PDS phase associated with the inability to upregulate genes required for respiratory metabolism, despite presenting intact mitochondrial DNA (Kitagaki *et al.*, 2007, Kitagaki *et al.*, 2009). In fact, these mutants display defective growth on non-fermentable carbon sources and enhanced frequency of petite formation. The mutant strain also displays decreased levels of the mitochondrial ETC complex IV (cytochrome *c* oxidase, COX) subunits Cox3p and Cox4p and this is correlated with decreased COX activity and oxygen consumption in *isc1*Δ cells (Almeida *et al.*, 2008, Kitagaki *et al.*, 2009, Barbosa *et al.*, 2011).

II. Isc1p-driven signalling in mitochondrial function and aging

Initial studies have demonstrated that Isc1p is implicated in the regulation of important cellular processes, namely responses to osmostress, heat stress and genotoxic agents. Almeida *et al.* have firstly reported that Isc1p is involved in the regulation of oxidative stress resistance, mitochondrial function and chronological lifespan (Almeida *et al.*, 2008). In fact, *isc1*Δ cells display shortened CLS, increased hydrogen peroxide sensitivity, which appear to be associated with mitochondrial dysfunction and increased iron uptake (Almeida *et al.*, 2008). Together with mitochondrial dysfunction, it promotes a vicious cycle of oxidative damage to biomolecules and contributes to cell death by apoptosis (Almeida *et al.*, 2008, Kitagaki *et al.*, 2009, Barbosa *et al.*, 2011).

In an attempt to dissect possible signalling pathways governing *isc1*Δ phenotypes, important downstream targets of Isc1p were identified and implicated in the regulation of mitochondrial function and CLS (Figure 1.11). Lipidomic analysis showed specific changes in sphingolipids in Isc1p-deficient cells during aging, including decreased dihydrosphingosine levels and an increase of very long chain base species, namely dihydro-C_{26}-ceramide and phyto-C_{26}-ceramide, the latter raising the possibility of activation of ceramide-dependent protein phosphatases. On this basis, it was shown that the deletion of *SIT4* gene, which encodes for the catalytic subunit of type 2A ceramide-activated protein phosphatases and presents considerable homology to human protein phosphatase 6 (PP6) (Bastians & Ponstingl, 1996), supresses mitochondrial dysfunctions, oxidative stress sensitivity and shortened CLS of Isc1p-deficient cells (Barbosa *et al.*, 2011) (Figure 1.11).

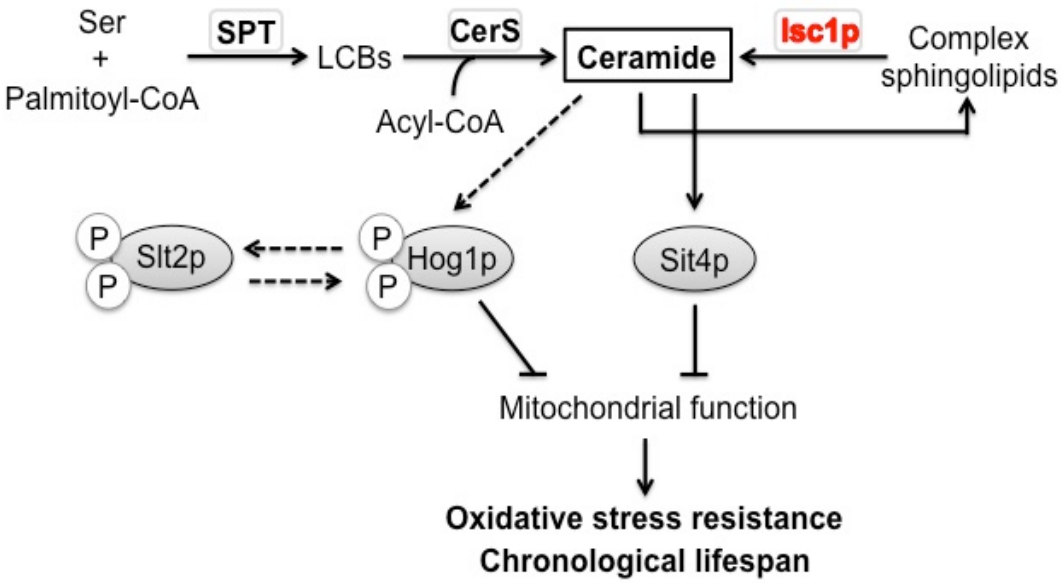


Figure 1.11. The sphingomyelinase Isc1p modulates ceramide signalling to regulate mitochondrial function and CLS in yeast. Isc1p-mediated signalling controls the activity of the ceramide-activated PP2A-like phosphatase Sit4p and the MAPK Hog1p, by regulating ceramide content in yeast.

Among other signalling pathways, the yeast high osmolarity-responsive (HOG) MAPK pathway, the HOG pathway, was also studied. It is activated in response to hyperosmotic stress via two independent osmosensing branches, the Sln1p and the Sho1p branches, which in turn control the phosphorylation of the

main effector, the MAPK Hog1p (Rep *et al.*, 2000, Alepuz *et al.*, 2001, Proft *et al.*, 2001, de Nadal *et al.*, 2003, de Nadal *et al.*, 2004, O'Rourke & Herskowitz, 2004, Proft *et al.*, 2006). It was demonstrated that sphingolipids are also able to modulate the HOG pathway in response to the inibition of the *de novo* biosynthetic pathway or depletion of ergosterol (Tanigawa *et al.*, 2012). Hog1p activation is deleterious for cells devoid of Isc1p since ceramide signalling increases Hog1p phosphorylation and the deletion of *HOG1* attenuates *isc1*Δ phenotypes (Barbosa *et al.*, 2012).

Scope of the thesis

Sphingolipids are important components of membranes in eukaryotes. More recently, they are recognized as important bioactive biomolecules implicated in the regulation of signalling transduction and important biological processes.

Several lines of evidence suggest an intricate interplay between sphingolipid metabolism and TOR signalling. In particular, TOR signalling is involved in the regulation of mitochondrial function, oxidative stress response and chronological lifespan (CLS) in yeast. Since *isc1*Δ cells exhibit mitochondrial dysfunction, hydrogen peroxide sensitivity and premature aging, we aimed to uncover the role of the nutrient-sensing Target Of Rapamycin Complex 1 (TORC1) and its downstream effector, the AGC protein kinase Sch9p, in these phenotypes.

The involvement of ceramide signalling in the regulation of important biological functions, such as autophagy and related processes, and mitochondrial quality control mechanisms, has been recently addressed in mammals. On this basis, we also aimed to determine how the Isc1p sphingomyelinase-driven ceramide signalling regulates macroautophagy, mitophagy and mitochondrial dynamics and evaluate its impact on longevity in yeast.

The deletion of *SIT4* extends lifespan in yeast by boosting mitochondrial function (mitochondrial catabolic derepression) and stress response. Although the PP2A-like protein phosphatase Sit4p functions downstream of Isc1p, the phosphatase is downregulated by TORC1 and was recently shown to control the turnover of complex sphingolipids in a Npr1p-dependent manner. However, how Sit4p integrates nutrient and sphingolipid signalling to regulate lifespan is not yet established. On this basis, we aimed to establish a functional association between Sit4p and sphingolipid metabolism and evaluate the impact on cell survival.

CHAPTER II

Science goes from question to question; big questions, and little, tentative answers. The questions as they age grow ever broader, the answers are seen to be more limited.

George Wald

Reduced TORC1 signalling abolishes mitochondrial dysfunctions and shortened chronological lifespan of Isc1p-deficient cells

Vitor Teixeira, Tânia C. Medeiros, Rita Vilaça, Pedro Moradas-Ferreira and Vítor Costa

Keywords: Isc1p; Tor1p; Sch9p; oxidative stress; chronological aging, mitochondrial function, ROS, ceramide.

Abstract

The target of rapamycin (TOR) is an important signalling pathway on a hierarchical network of interacting pathways regulating central biological processes, such as cell growth, stress response and aging. Several lines of evidence suggest a functional link between TOR signalling and sphingolipid metabolism. Here, we report that the TORC1-Sch9p pathway is activated in cells lacking Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase 2. The deletion of *TOR1* or *SCH9* abolishes the premature aging, oxidative stress sensitivity and mitochondrial dysfunctions displayed by *isc1*Δ cells and this is correlated with the suppression of the autophagic flux defect exhibited by the mutant strain. The protective effect of *TOR1* deletion, as opposed to that of *SCH9* deletion, is not associated with the attenuation of Hog1p hyperphosphorylation, which was previously implicated in *isc1*Δ phenotypes. Our data support a model in which Isc1p regulates mitochondrial function and chronological lifespan in yeast through the TORC1-Sch9p pathway although Isc1p and TORC1 also seem to act through independent pathways, as *isc1*Δ*tor1*Δ phenotypes are intermediate to those displayed by *isc1*Δ and *tor1*Δ cells. We also provide evidence that TORC1 downstream effectors, the type 2A protein phosphatase Sit4p and the AGC protein kinase Sch9p, integrate nutrient and stress signals from TORC1 with ceramide signalling derived from Isc1p to regulate mitochondrial function and lifespan in yeast. Overall, our results show that TORC1-Sch9p axis is deregulated in Isc1p-deficient cells, contributing to mitochondrial dysfunction, enhanced oxidative stress sensitivity and premature aging of *isc1*Δ cells.

2.1. Introduction

Sphingolipids are ubiquitous structural components of eukaryotic cell membranes, and their bioactive metabolites (sphingosine, sphingosine-1-phosphate, ceramide, ceramide-1-phosphate and lyso-sphingomyelin) are known to act as second messengers in the transduction and regulation of signalling pathways (Dickson & Lester, 1999, Jenkins & Hannun, 2001, Spiegel & Milstien, 2003, Dickson, 2008, Hannun & Obeid, 2008, van Meer *et al.*, 2008). Sphingosine (and related sphingoid bases) and ceramide are involved in the regulation of actin cytoskeleton organization, endocytosis, degradation of nutrient permeases, apoptosis, cell senescence and cell cycle arrest whereas sphingosine-1-phosphate plays a key role in proliferation, mitogenesis, cell migration, cell survival and inflammation (in higher eukaryotes) (Hannun & Obeid, 2008). Thus, subtle variations on the relative amounts of sphingosine-1-phosphate and sphingosine/ceramide are expected to determine cell fate in response to environmental or metabolic stresses. The importance of sphingolipids is recognized by the fact that sphingolipid signalling is implicated in the pathobiology of human cancers and other human diseases such as diabetes and heart disease, microbial infections, neurological and immune dysfunctions (Kolter & Sandhoff, 2006, Ozbayraktar & Ulgen, 2009, Kolter, 2011, Hla & Dannenberg, 2012, Young *et al.*, 2013).

Sphingolipids metabolism and their route of synthesis are highly conserved from yeast to mammalian cells. Studies using the budding yeast *Saccharomyces cerevisiae* have served in many ways to foster our understanding of sphingolipid dynamics and their role in the regulation of cell cycle, cell integrity, endocytosis, cytoskeleton dynamics and protein turnover (Dickson, 2008, Hannun & Obeid, 2008). Additionally, sphingolipids have been implicated in the regulation of stress responses and longevity. For instance, yeast mutants lacking Ydc1p (dihydroceramidase) are characterized by increased chronological lifespan (CLS) whereas the overexpression of *YDC1* triggers mitochondria and vacuolar fragmentation, apoptosis and accelerated aging in yeast (Aerts *et al.*, 2008). Genes involved in sphingolipid metabolism (*LAG1*, *YPC1*, *YSR3*, *IPT1*, and *LCB5*) show variable expression in senescent and apoptotic cells (Laun *et al.*, 2005).

More recently, it was demonstrated that the downregulation of sphingolipid synthesis increases yeast CLS in part due to a reduction in long-chain bases (LCBs) mediated activation of Sch9p, the yeast homologue of mammalian ribosomal S6K protein kinase (Huang *et al.*, 2012). Furthermore, ceramide synthase (Lag1p) and LCB kinase (Lcb4p) activities decrease upon entry into the stationary phase, leading to a large increase in the levels of LCBs (Lester *et al.*, 2013).

We have previously demonstrated that Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase-2 (nSMase2) responsible for the hydrolysis of complex inositol phosphosphingolipids to produce ceramide, is implicated in oxidative stress resistance and chronological lifespan in yeast. Isc1p-deficient cells display shortened CLS, oxidative stress sensitivity and impaired redox and iron homeostasis (Almeida *et al.*, 2008). Analogous to the role of ceramide and ceramide-activated protein phosphatases in regulating mammalian cell apoptosis, Isc1p acts upstream of Sit4p, the catalytic subunit of protein phosphatase related to type 2A protein phosphatases (PP2A) in yeast. Indeed, *SIT4* deletion restores mitochondrial function of *isc1*Δ cells, increasing oxidative stress resistance and extending CLS (Barbosa *et al.*, 2011). The activation of the HOG (High Osmolarity Glycerol) pathway is also deleterious for *isc1*Δ cells since ceramide signalling increases the phosphorylation of the Hog1p mitogen activated protein kinase (MAPK) and the deletion of *HOG1* attenuates the phenotypes of Isc1p-deficient cells (Barbosa *et al.*, 2012).

Recent studies also link ceramide to other important signalling pathways involved in the regulation of cell growth and survival, namely the TOR (Target of Rapamycin) pathway. This pathway is highly conserved among organisms, ranging from flies, nematodes, protozoa alongside with mammals (Raught *et al.*, 2001, Dann & Thomas, 2006, de Virgilio & Loewith, 2006, Laplante & Sabatini, 2012, Johnson *et al.*, 2013, Markaki & Tavernarakis, 2013). In *S. cerevisiae*, the TOR pathway is controlled by two Ser/Thr protein kinases, Tor1p and Tor2p, which assemble into two protein complexes with distinct subunit composition and regulatory roles (Loewith *et al.*, 2002, Kim & Guan, 2011, Loewith & Hall, 2011). The rapamycin-sensitive TOR complex 1 (TORC1) contains either Tor1p or Tor2p and is mostly associated with the regulation of cell growth (nutrient sensing), autophagy, ribosomal and protein turnover and cell proliferation (Evans *et al.*,

2011, Kim & Guan, 2011). The TOR complex 2 (TORC2) contains Tor2p, but not Tor1p, and mediates the proper maintenance of the cell cytoskeleton (Cybulski & Hall, 2009) and was recently implicated in the regulation of ceramide biosynthesis by a Ypk2p-dependent mechanism (Aronova *et al.*, 2008). Furthermore, TORC2 indirectly modulates Isc1p turnover through the phosphorylation and activation of Slm1p and Slm2p (Tabuchi *et al.*, 2006).

The TORC1 pathway has also been linked to mitochondrial function and yeast CLS (Bonawitz *et al.*, 2007, Pan *et al.*, 2011). In fact, the deletion of *TOR1* or pharmacological inhibition of TORC1 with rapamycin extends CLS in yeast and other organisms (Powers *et al.*, 2006, Bonawitz *et al.*, 2007, Kaeberlein & Kennedy, 2011). In yeast, TORC1 is active during early stages of growth and represses the induction of stress responses and entry into the stationary phase, in part by inhibiting the Rim15p protein kinase and consequently the translocation of Msn2p/4p and Gis1p transcription factors into the nucleus to induce adaptive response required for CLS extension (Wanke *et al.*, 2005, Wei *et al.*, 2008). Reducing TORC1 signalling at early stages of growth extends CLS by an intrinsic mechanism involving enhanced mitochondrial membrane potential and superoxide production. This in turn induces an adaptive response that contributes to decrease ROS production in the stationary phase and promote longevity in yeast (Pan *et al.*, 2011). Moreover, reduced TORC1 signalling derepresses Rim15p and triggers the expression of genes regulated by the mitochondrial signalling pathway known as the retrograde response (Komeili *et al.*, 2000, Dilova *et al.*, 2004, Liu & Butow, 2006) as well as stress-related genes under the control of Msn2p/Msn4p (Beck & Hall, 1999, Wei *et al.*, 2008).

Some authors have identified downstream targets of TORC1 involved in the regulation of stress response and aging, namely the AGC protein kinase Sch9p and the Sit4p protein phosphatase. Apart from sensing nutrient and stress signals from TORC1, both proteins also regulate CLS by integrating sphingolipid signalling. In addition to phosphorylation in the C-terminus mediated by TORC1, Sch9p is phosphorylated in a Thr570 residue in the activation loop by Pkh1/2p protein kinases, homologues of mammalian phosphoinositide-dependent protein kinase 1 (PDK1), in response to LCBs (Voordeckers *et al.*, 2011, Huang *et al.*, 2012). On the other hand, Sit4p is downregulated by TORC1 in a Tip41p/Tap42p manner (Di Como & Arndt, 1996, Jacinto *et al.*, 2001) but is also activated by

ceramide and functions downstream of Isc1p (Barbosa *et al.*, 2011). How this complex network of interacting pathways regulates CLS remains poorly characterized.

Thus, we postulated that TORC1 may be deregulated in *isc1*Δ cells. In this study, we show that TORC1 activation impairs oxidative stress resistance, mitochondrial function and chronological lifespan in *isc1*Δ cells, suggesting that Isc1p regulates mitochondrial function and yeast chronological lifespan, either through the TORC1 pathway or in a functionally related pathway parallel to TORC1. The suppression of *isc1*Δ phenotypes by reduced TORC1 signalling is not associated with the attenuation of Hog1p hyperphosphorylation. Our data also support a model in which TORC1 downstream effectors Sit4p and Sch9p act as physiological hubs integrating nutrient and ceramide signalling driven by Isc1p.

2.2. Results

2.2.1. The deletion of *TOR1* and *SCH9* extends chronological lifespan and increases oxidative stress resistance of *isc1*Δ cells

To assess changes in TORC1 signalling associated with Isc1p deficiency, we have monitored TORC1 activity *in vivo* by assessing TORC1-dependent Sch9p phosphorylation at the C-terminus (Urban *et al.*, 2007). The results show that Sch9p is hyperphosphorylated in *isc1*Δ mutants when compared to parental cells, indicative of increased TORC1 activity (Figure 2.1.A-B). Apart from TORC1, Sch9p is also phosphorylated by Pkh1/2p in response to LCBs. Since basal levels of phytosphingosine (PHS) are increased in *isc1*Δ cells during aging (Barbosa *et al.*, 2011), we also assessed the Pkh1/2p-dependent phosphorylation of Sch9p on the Thr570 residue. The results show that the levels of Sch9p-phospho-Thr570 were similar in parental and *isc1*Δ cells (Figure 2.S1).

This led us to postulate that TORC1 activation may contribute to *isc1*Δ phenotypes. To test this hypothesis, we then evaluated if the deletion of *TOR1* or *SCH9* could abolish the premature aging and hydrogen peroxide sensitivity of *isc1*Δ cells. In agreement with previous reports, *isc1*Δ cells presented a shortened CLS compared to parental cells (Figure 2.2.A). Although the acidification of the

growth medium due to acetic acid production decreases yeast lifespan (Burtner *et al.*, 2009), it is unlikely that it contributes to the shortened CLS of *isc1*Δ cells since *ISC1* deletion increases acetic acid resistance (Rego *et al.*, 2012). As expected, *tor1*Δ and *sch9*Δ cells exhibited an increased lifespan (Figure 2.2.A).

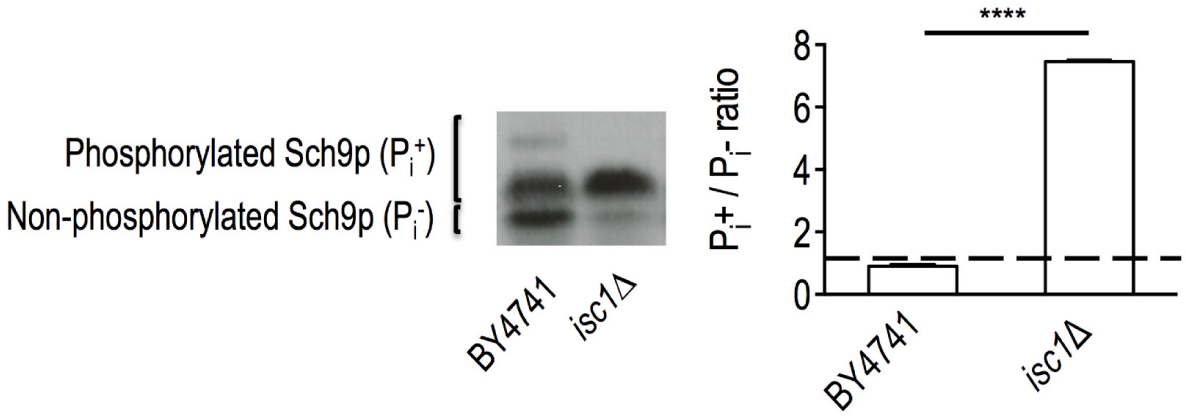


Figure 2.1. Isc1p-deficient cells exhibit enhanced TORC1 activity *in vivo.* The TORC1-dependent C-terminal phosphorylation of Sch9p was used to assess TORC1 activity *in vivo*. *S. cerevisiae* BY4741 and *isc1*Δ cells transformed with pJU676 (expressing *SCH9*-5HA) were grown in SC-medium to the exponential phase and NTCB-treated protein extracts were analyzed by immunoblotting using anti-HA antibody, as described in *Experimental Procedures*. A representative blot out of three is shown. Quantification of band intensities was performed by densitometry. The ratio between phosphorylated and unphosphorylated Sch9p (P_i^+/P_i^-) is shown. Values are mean ± SD of at least three independent experiments. ****p<0.0001.

The effect of *SCH9* deletion was significantly lower to that reported by other groups (Fabrizio *et al.*, 2001). This probably arises from differences in the growth medium composition, in particular amino acid concentration. Indeed, *sch9*Δ mutants display increased lifespan when cells are grown in media supplemented with a 3.5-fold excess of amino acids but present shortened lifespan than parental cells when grown in media with 0.5-fold aminoacid content (Wu *et al.*, 2013). Importantly, the deletion of *TOR1* or *SCH9* in *isc1*Δ cells significantly extended the CLS of this mutant but the *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ double mutants exhibited a shorter CLS compared to that of *tor1*Δ and *sch9*Δ cells, respectively (Figure 2.2.A).

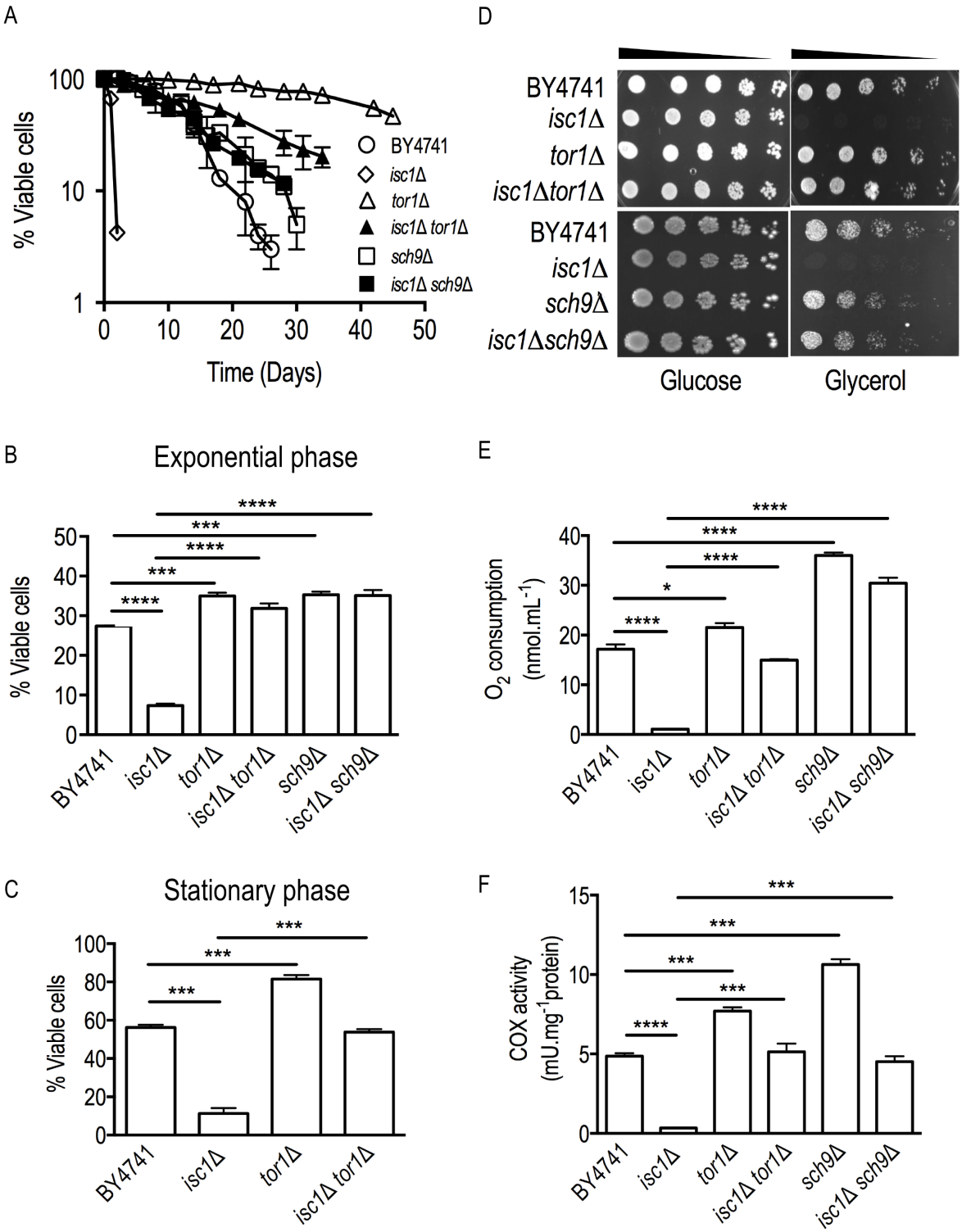


Figure 2.2. The deletion of *TOR1* and *SCH9* abolishes the shortened CLS, oxidative stress sensitivity and mitochondrial dysfunctions displayed by *isc1*Δ cells.

A. *S. cerevisiae* BY4741, *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells were grown in SC-medium and kept in the medium at 26°C. The viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units at time *T* in relation to T_0. Values are mean ± SD of at least three independent

experiments. (**B, C**). Yeast cells were grown in SC-medium to the exponential phase (B) or stationary phase (48 hours after exponential phase) (C) and exposed to 1.5 mM H_2O_2 for 60 min (B) or 300 mM H_2O_2 for 30 min (C). Cell viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units (treated cells versus untreated cells). Values are mean ± SD of at least three independent experiments. ****p<0.0001. ***p<0.001. **D.** *S. cerevisiae* BY4741, *isc1*Δ*, tor1*Δ and *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells were grown in SC-medium to the exponential phase and then diluted to OD_{600}=0.1. Fivefold dilution series were spotted on YPD (glucose) or YPG (glycerol) medium and cells were grown at 26°C for 4 days**. E.** Oxygen consumption rate was measured in cells grown to PDS phase, as described in *Experimental Procedures.* Values are mean ± SD of at least three independent experiments. ****p<0.0001; *p<0.05. **F.** Cytochrome *c* oxidase (COX) activity was determined in cells grown to the PDS phase. Cells were lysed and enzymatic activity was measured as described in *Experimental Procedures.* Values are mean ± SD of at least three independent experiments. ****p<0.0001; ***p<0.001

To assess oxidative stress resistance, cells were grown to the exponential or early stationary phase and treated with H_2O_2. Consistent with published data (Wei *et al.*, 2009), *tor1*Δ and *sch9*Δ cells were more resistant to oxidative stress than parental cells (Figure 2.2.B-C). The deletion of *TOR1* or *SCH9* suppressed the hydrogen peroxide sensitivity of *isc1*Δ cells (Figure 2.2.B-C). Similar results were obtained by the pharmacological inhibition of TORC1 using rapamycin (Figure 2.S2). These results implicate TORC1-Sch9p activation in the premature aging and oxidative stress sensitivity of Isc1p-deficient cells.

2.2.2. Reduced TORC1 signalling enhances mitochondrial coupled respiration in *isc1*Δ cells

It was previously shown that *tor1*Δ cells have an extended CLS in part associated with improved and better coupled mitochondrial respiration at early stages of growth, which ultimately preconditions yeast to better survive on the stationary phase (Pan *et al.*, 2011). Therefore, we evaluated if the deletion of *TOR1* and *SCH9* could improve mitochondrial fitness of *isc1*Δ cells, eventually contributing to CLS extension observed in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ double mutants. To address this hypothesis, we analyzed cell growth in medium

containing glycerol, a non-fermentable carbon source, as well as oxygen consumption and cytochrome *c* oxidase (COX) activity. It was observed that the growth defect of *isc1*Δ cells on glycerol medium was suppressed upon the deletion of *TOR1* or *SCH9* (Figure 2.2.D). As expected, the deletion of *ISC1* almost completely abolished oxygen consumption and COX activity at the post-diauxic shift (PDS; respiratory) phase (Figure 2.2.E-F). In *tor1*Δ and *sch9*Δ cells, both oxygen consumption and COX activity were increased when compared to parental cells. Notably, *TOR1 and SCH9* disruption suppressed the defects observed in *isc1*Δ cells: both oxygen consumption and COX activity increased in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells, although to levels lower than those observed in *tor1*Δ and *sch9*Δ cells, respectively (Figure 2.2.E-F).

To assess if enhanced mitochondrial respiration coupling contributes to the lifespan extension of *isc1*Δ cells imposed by reduced TORC1 signalling, CLS was analyzed in cells treated with 2,4-dinitrophenol (DNP), which decreases mitochondrial membrane potential and uncouples electron transport from ATP synthesis. Untreated Isc1p-deficient cells exhibited a shortened CLS that was not affected by exposure to DNP (Figure 2.3), providing strong evidence that mitochondrial dysfunction largely contributes to *isc1*Δ phenotypes. In *tor1*Δ mutants, DNP significantly affected cell viability (e.g., it decreased 40% in DNP-treated vs control cells aged for 2 days). However, the detrimental effect of DNP in *tor1*Δ mutants was lower to the observed in parental cells (in this strain, the viability of cells treated with DNP and aged for 2 days decreased 68%). This is consistent with published data showing that *tor1*Δ cells present better coupled respiration when compared to parental cells (Pan *et al.*, 2011), and therefore are able to mount a more effective adaptive response to counteract DNP effects. The deletion of *TOR1* in *isc1*Δ cells extended CLS (in cells untreated with DNP) and this effect appears to be correlated with an increased coupled respiration imparted by reduced TORC1 signalling since DNP treatment decreased cell viability in such conditions. The *isc1*Δ*tor1*Δ double mutant presented an intermediate phenotype between parental and *tor1*Δ cells since, at day 2 of aging, parental, *tor1*Δ and *isc1*Δ*tor1*Δ cells presented 32%, 60% and 40% cell viability, respectively. Taking together, we claim that improved mitochondrial fitness (increased coupled respiration) promoted by the deletion of *TOR1 per se* extends CLS in *isc1*Δ cells, in similar fashion as observed for the effect of *TOR1* deletion in parental cells.

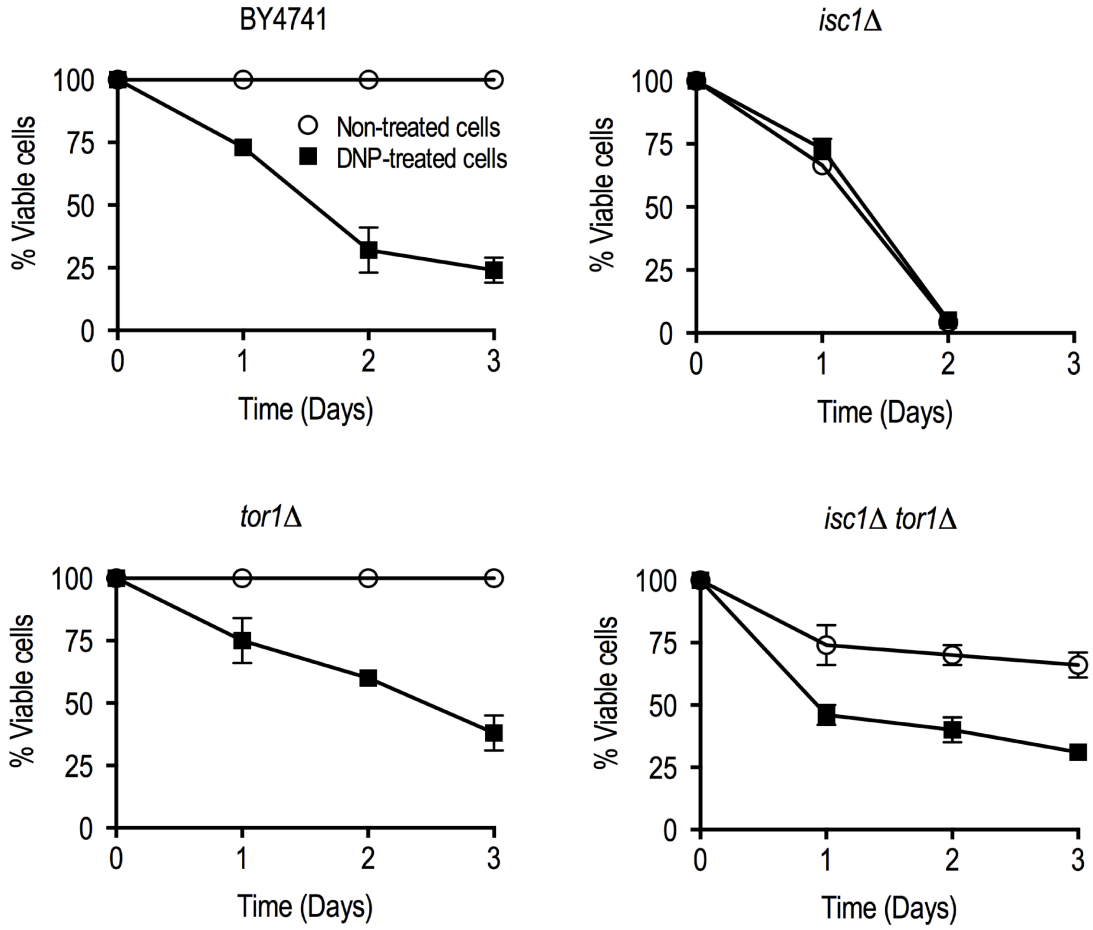


Figure 2.3. Increased mitochondrial coupled respiration imparted by reduced TORC1 signalling extends lifespan in *isc1*Δ cells. *S. cerevisiae* BY4741, *isc1*Δ*, tor1*Δ and *isc1*Δ*tor1*Δ cells were grown in SC-medium to the PDS phase, treated with 10 μM 2,4-dinitrophenol (DNP; square) or vehicle (DMSO; circle) and kept in the medium at 26°C. The viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units at time *T* in relation to T_0. Values are mean ± SD of at least three independent experiments. The linear plot was used for better visual assessment.

2.2.3. Hyperpolarization and fragmentation of the mitochondrial network in *isc1*Δ cells are suppressed by *TOR1* or *SCH9* deletion

To get further insights into alterations in mitochondrial function operating on *isc1*Δ cells, we assessed the mitochondrial membrane potential ($\Delta\psi_m$), a parameter that has been used to monitor changes on bioenergetics as a key indicator of cell health or injury (Nicholls, 2004).

For this purpose, yeast cells were labeled with a mitochondria-specific voltage-dependent dye, 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], which aggregates and preferentially accumulates into functional mitochondria, and analyzed by flow cytometry (Figure 2.4.A).

At PDS phase, cells lacking Isc1p displayed enhanced $\Delta\psi_m$ when compared to parental cells, which is consistent with mitochondrial hyperpolarization. In contrast, *tor1*Δ and *sch9*Δ cells had a slightly lower $\Delta\psi_m$, which has been associated with mild mitochondrial uncoupling (Pan *et al.*, 2011). Both *TOR1* and *SCH9* disruption in *isc1*Δ cells reversed the mitochondrial hyperpolarization and decreased the $\Delta\psi_m$ to values similar to those observed in the respective single mutants. This probably increases cell survival in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells, since *isc1*Δ cells die by caspase-dependent apoptosis upon oxidative stress and during cell aging (Almeida *et al.*, 2008) and mitochondrial hyperpolarization has been associated with the activation of a mitochondrial dependent apoptotic pathway, which initially involves a transient hyperpolarization followed by depolarization of the mitochondrial membrane and release of cytochrome *c* from the mitochondria into the cytosol (Kroemer *et al.*, 2007).

The mitochondrial membrane potential plays a key role in the regulation of mitochondrial morphology and alterations on this parameter were demonstrated to impact on mitochondrial dynamics (Legros *et al.*, 2002, Detmer & Chan, 2007, Berman *et al.*, 2008, Oliveira, 2012). To assess changes in mitochondrial network dynamics, yeast cells expressing a mitochondrial-targeted DsRed protein were analyzed by fluorescence microscopy. At the exponential phase, the mitochondrial network was not yet fully developed and no significant differences were observed between the parental and *isc1*Δ cells (Figure 2.4.B). This was expected since cells are undergoing fermentative growth at this phase. However, in the PDS phase, loss of Isc1p led to the formation of a typical punctuate pattern contrasting with the tubular and well-organized network observed in healthy parental cells (Figure 2.4.B). This structural alteration has been associated with mitochondrial fragmentation and observed in cells undergoing apoptotic cell death. The normal tubular mitochondrial network was restored upon disruption of *TOR1* or *SCH9* in *isc1*Δ cells (Figure 2.4.B), suggesting that TORC1 and its downstream target Sch9p are also implicated in the regulation of mitochondrial dynamics by promoting network fragmentation.

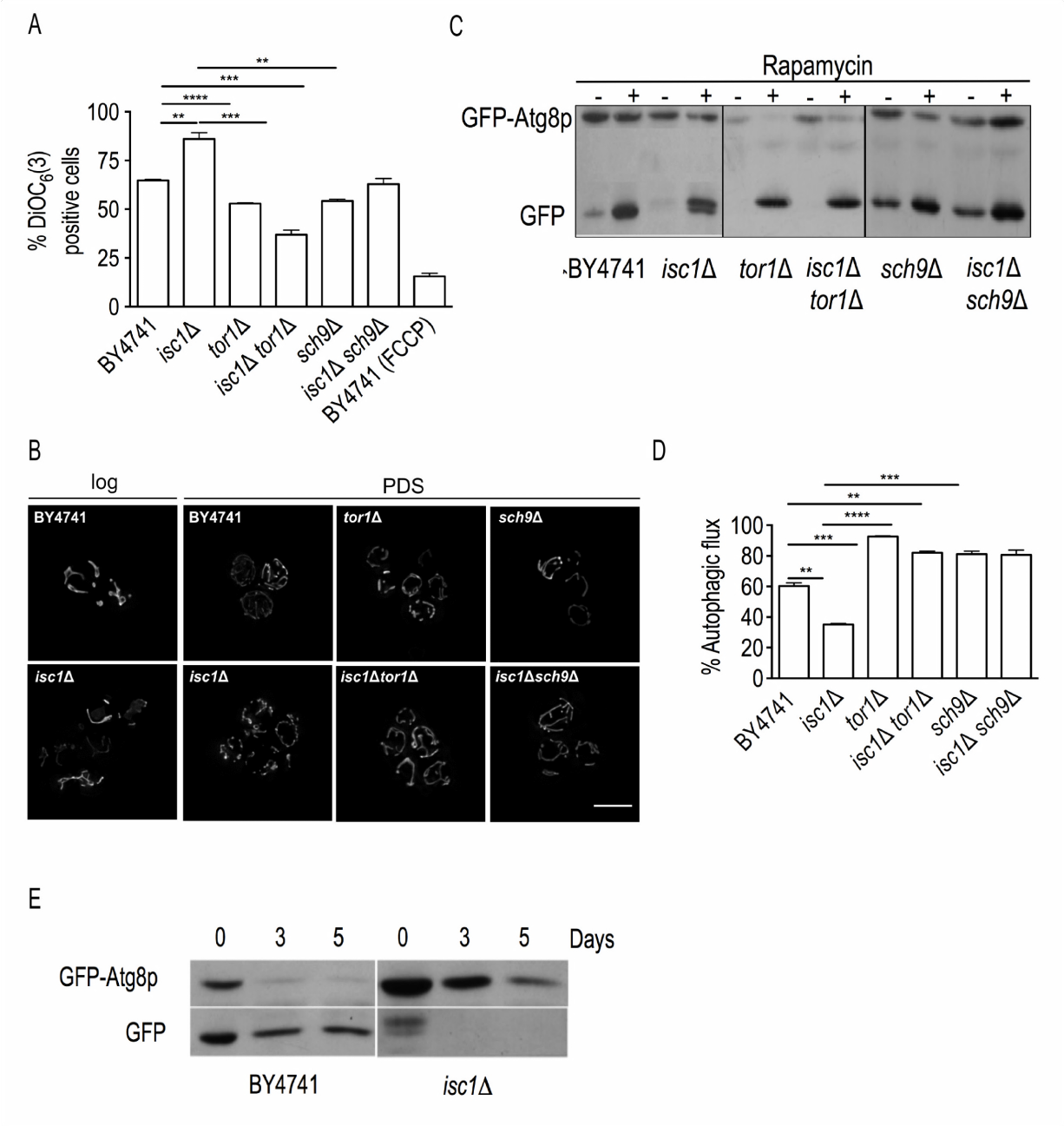


Figure 2.4. The mitochondrial membrane hyperpolarization and decreased autophagic flux contribute to mitochondrial dysfunction and impairment of mitochondrial dynamics in *isc1*Δ cells.

A. *S. cerevisiae* BY4741, *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells were grown in SC-medium to the PDS phase, stained with the potential-sensitive dye 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] for 30 min and analyzed by flow cytometry. Treatment of the parental strain (BY4741) with FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) was used as a positive control (depolarizing event). Values are mean ± SD of at least three independent experiments. ****p<0.0001; ***p<0.001; **p<0.01. **B.** Yeast cells transformed with pYX222-mtDsRed were grown to

the exponential and PDS phases and analyzed by fluorescence microscopy, as described in *Experimental Procedures*. Live cells were visualized by fluorescence microscopy. A representative experiment out of three is shown. Scale bar: 5 µm. **C.** *S. cerevisiae* BY4741, *isc1*Δ*, tor1*Δ and *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells carrying pRS416 GFP-*ATG8* were grown to the exponential phase in SC-medium and treated with either rapamycin (200 ng/mL) or DMSO (vehicle) for 3 h. Proteins were analyzed by immunoblotting, using anti-GFP antibody. **D.** The autophagic flux was calculated by the ratio between the free GFP signal and the sum of free GFP and GFP-Atg8p signals. Values are mean ± SD of at least three independent experiments ****p<0.0001; ***p<0.001; **p<0.01. **E**. *S. cerevisiae* BY4741 and *isc1*Δ cells carrying pRS416 GFP-*ATG8* were grown to PDS phase, washed twice with water and then maintained in water. Proteins were analyzed by immunoblotting, using anti-GFP antibody.

Previous studies have demonstrated that autophagy has an important role in maintaining proper mitochondrial function and dynamics since autophagy-defective mutants present severe mitochondrial dysfunctions (Suzuki *et al.*, 2011, Aung-Htut *et al.*, 2013). In particular, the regulation of the mitochondrial membrane potential appears to be crucial to regulate autophagic flux. In addition, accumulating evidence show that defects in autophagy compromise mitochondrial dynamics (Lee *et al.*, 2012). Since *isc1*Δ cells present similar phenotypic features and TORC1, a negative regulator of autophagy, is activated in this mutant strain, we evaluated if these cells present autophagy defects. For this purpose, we have monitored the processing of GFP-Atg8p. Once autophagy is induced, GFP-Atg8p is recruited to drive autophagosome biogenesis and then delivered to the vacuole inside the autophagic body. Whereas Atg8p is degraded by resident vacuolar hydrolases, the GFP moiety is relatively resistant to proteolysis. Therefore, the appearance of free GFP signal is indicative of autophagy induction. To induce autophagy, yeast cells were treated with rapamycin. Under these conditions, the autophagic flux was significantly lower in Isc1p-deficient cells (35%) when compared to the parental strain (60%) (Figure 2.4.C-D). Notably, a slower migrating band (above free GFP) was observed in *isc1*Δ cells, both under basal conditions and upon rapamycin treatment (Figure 2.S3). It probably results from an incomplete or aberrant processing of GFP-Atg8p, possibly due to vacuolar dysfunction (defective Pep4p-dependent proteolytic activity) or alterations in vacuolar morphology upon deletion of *ISC1* (Seeley *et al.*, 2002). In *isc1*Δ*tor1*Δ

and *isc1*Δ*sch9*Δ double mutants, the autophagic flux increased to values close to those observed for the respective *tor1*Δ and *sch9*Δ single mutants (Figure 2.4.C-D). It should be noted that rapamycin is predicted to have still some effect on *tor1*Δ cells because there is yet some functional TORC1 signalling. In fact, TORC1 complex is sensitive to rapamycin due to binding of FKBP-rapamycin complex to subunits of TORC1. Thus, TORC1 is functional (although with reduced activity) in *tor1*Δ cells since it still contains Tor2p (which may replace Tor1p) in its composition. These results implicate TORC1 and its downstream effector, Sch9p, in the deregulation of autophagy, possibly contributing to mitochondrial network fragmentation and impairment of oxidative stress resistance and CLS in *isc1*Δ cells.

To provide further evidence that autophagy is impaired in *isc1*Δ cells, we have also monitored the autophagic flux during chronological aging. The rate of viability loss during aging of this mutant is very high when cells are grown in SC-medium (Figure 2.2.A). Thus, to avoid unspecific changes that may occur due to cell death, we assessed autophagy under conditions of calorie restriction (cells grown to PDS phase, washed and maintained in water overtime). We have previously shown that calorie restriction increases CLS in both *isc1*Δ and parental cells, but *isc1*Δ mutants still exhibit a premature aging phenotype (Barbosa *et al.*, 2011). The results show that the autophagic flux increased in parental cells aged for 3-5 days, but it was significantly reduced in *isc1*Δ cells (Figure 2.4.E). Overall, the data suggests that autophagy is impaired in this mutant strain.

2.2.4. *TOR1* and *SCH9* deletion in *isc1*Δ cells decreases ROS levels, catalase A deficiency and apoptotic cell death

Apoptosis and aging has been extensively associated with enhanced ROS production (Raftopoulou, 2005, Simm & Brömme, 2005, Kregel & Zhang, 2007, Marchi *et al.*, 2012). Thus, the improvement of mitochondrial function and/or antioxidant defenses may decrease mitochondrial ROS production or increase its detoxification, leading to CLS extension. To test this hypothesis, ROS levels were measured by flow cytometry using early stationary phase cells stained with dihydroethidium (DHE), a molecular probe sensitive to superoxide radicals. The results show that ROS levels were low in parental, *tor1*Δ and *sch9*Δ cells but

approximately 50% of *isc1*Δ cells were DHE-positive (Figure 2.5.A). In *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ mutants, ROS levels were higher than in parental cells but significantly lower when compared to Isc1p-deficient cells (by approximately one-half), suggesting that *TOR1* and *SCH9* deletion increases survival in *isc1*Δ cells by decreasing ROS generation.

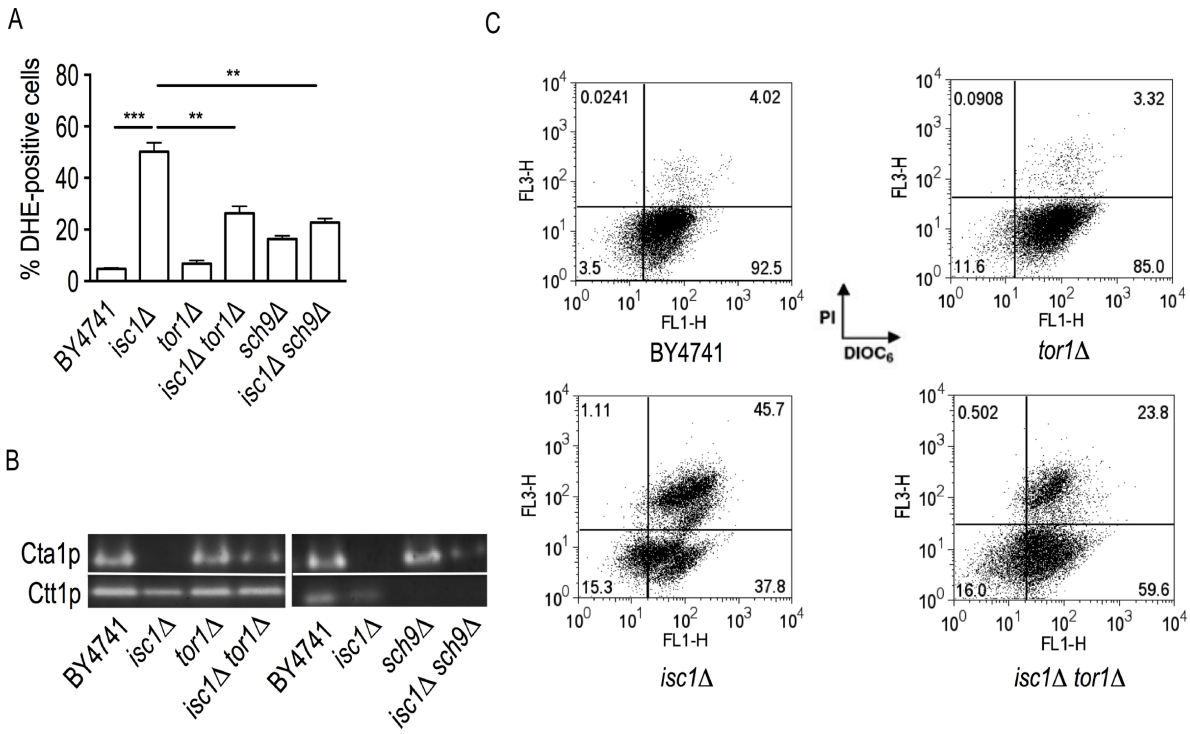


Figure 2.5. The deletion of *TOR1* or *SCH9* decreases ROS production, attenuates catalase A deficiency and diminishes apoptotic cell death in *isc1*Δ cells.

A. *S. cerevisiae* BY4741, *isc1*Δ*, tor1*Δ and *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells were grown to early stationary phase (day 1 in the CLS assay), stained with the dihydroethidium (DHE) for 10 min and analyzed by flow cytometry. Values are mean ± SD of at least three independent experiments. ***p<0.001; **p<0.01. **B.** Yeast cells were grown to the PDS phase and catalase activity was detected *in situ* after non-denaturing polyacrylamide gel electrophoresis, using the H_2O_2/peroxidase system, as described in *Experimental* Procedures. A representative experiment out of three is shown. **C.** BY4741, *isc1*Δ*, tor1*Δ and *isc1*Δ*tor1*Δ cells were double stained with $DiOC_6(3)$ and PI (propidium iodide) and analyzed by flow cytometry, as described in *Experimental Procedures*. Representative histograms are shown.

Increased ROS levels have been associated with homeostatic imbalance partially dictated by impaired cellular antioxidant defences. Hence, we

hypothesized that the improvement of the mitochondrial function and dynamics in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells may contribute to upregulate antioxidant defence mechanisms and decrease ROS levels during the aging process. It was previously shown that *isc1*Δ cells fail to induce *CTA1* gene expression in the PDS phase (Kitagaki *et al.*, 2009) and display a low activity of Cta1p (Barbosa *et al.*, 2011), the catalase A form present in mitochondria and peroxisomes. Moreover, *CTA1* overexpression partially suppresses *isc1*Δ phenotypes (Barbosa *et al.*, 2011). Our results show that Cta1p activity was partially restored in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells (Figure 2.5.B). In *sch9*Δ and *isc1*Δ*sch9*Δ cells, Ctt1p (cytosolic catalase) activity was not detected, which is consistent with the fact that the Sch9p kinase is directly or indirectly involved in the transcriptional control of *CTT1* expression in yeast (Pascual-Ahuir & Proft, 2007). The analysis of superoxide dismutase activity did not reveal changes in Sod1p (cytosolic form) or Sod2p (mitochondrial form) activity upon the deletion of *TOR1* or *SCH9* in *isc1*Δ cells (data not shown).

To evaluate if the decrease in ROS production and enhancement of antioxidant defenses (Cta1p) contribute to decrease apoptotic cell death in *isc1*Δ*tor1*Δ mutants, early stationary phase cells were labeled with $DiOC_6(3)$/propidium iodide (PI). The analysis of yeast cells by flow cytometry allows the definition of four distinct populations: healthy cells ($DiOC_6(3)$-positive/PI-negative), early apoptosis ($DiOC_6(3)$-negative/PI-negative), late apoptosis ($DiOC_6(3)$-positive/PI-positive) and necrosis ($DiOC_6(3)$-negative/PI-positive). The results (Figure 2.5.C) clearly show that the large majority of parental and *tor1*Δ cells remained healthy whereas 61% of the *isc1*Δ cell population was already undergoing early (15%) or late (46%) apoptosis. In the *isc1*Δ*tor1*Δ double mutant, however, a significant decrease of apoptotic markers was observed, particularly at late stages of apoptosis, where a reduction of approximately 50% (from 46% to 24%) was detected.

2.2.5. *SCH9* deletion but not reduced TORC1 signalling in *isc1*Δ cells attenuates Hog1p activation

The hyperactivation of the HOG signalling pathway was previously implicated in the premature aging and mitochondrial dysfunction exhibited by

Isc1p-deficient cells (Barbosa *et al.*, 2012). Thus, we hypothesized that the suppression of *isc1*Δ phenotypes by *TOR1* and *SCH9* deletion could be associated with the modulation of the HOG pathway. To address this question, Hog1p phosphorylation was monitored by Western blotting using an anti-phospho-p38 antibody that recognizes dually phosphorylated Hog1p, the active form of this kinase (Millar *et al.*, 1995, Smith *et al.*, 2004). As illustrated in Figure 2.6.A, Hog1p phosphorylation was increased in *isc1*Δ cells when compared to parental cells. In contrast, phosphorylated Hog1p could not be detected in *tor1*Δ cells. Notably, Hog1p phosphorylation levels in *isc1*Δ*tor1*Δ cells were even higher to those detected in *isc1*Δ cells, suggesting a functional interplay between the HOG and TORC1 signalling pathways in *isc1*Δ cells.

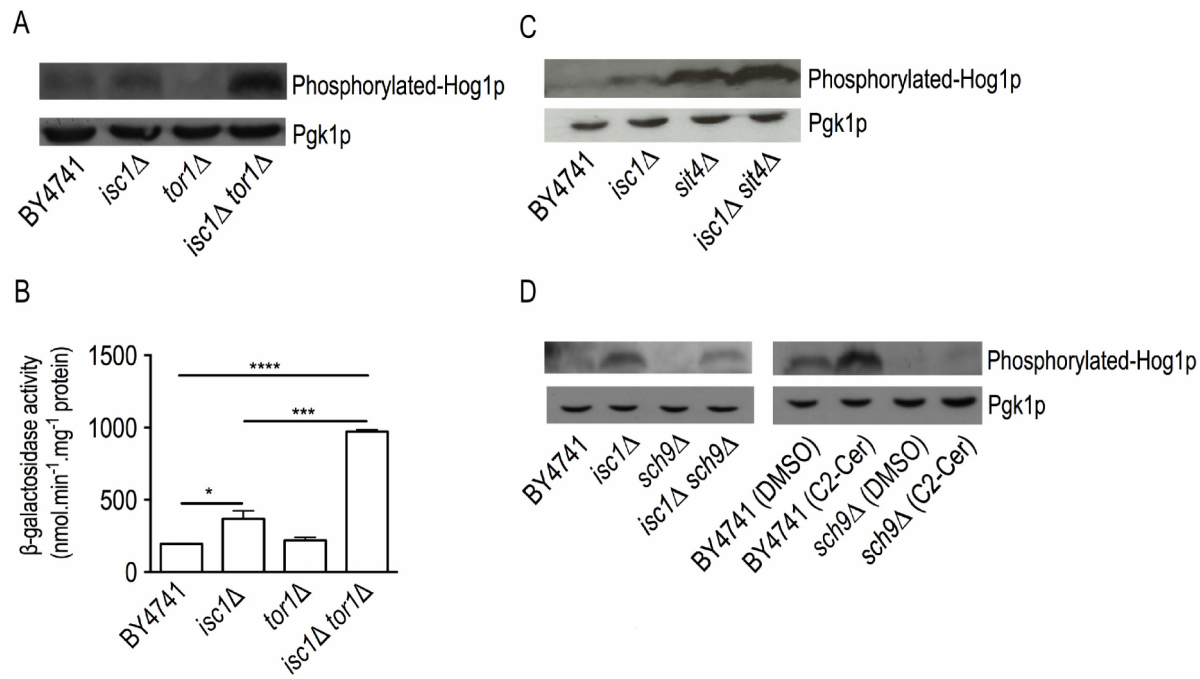


Figure 2.6. The deletion of *TOR1* increases Hog1p phosphorylation without affecting its cytosolic localization whereas *SCH9* disruption diminishes Hog1p phosphorylation in *isc1*Δ cells.

A. Hog1p activation was monitored in BY4741, *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ cells by immunoblotting, using anti-phospho-p38 antibody (top panel) that detects the phosphorylated form of Hog1p, or anti-Pgk1p (loading control) as primary antibodies. A representative blot out of three is shown. **B.** *S. cerevisiae* BY4741, *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ cells expressing the consensus Rlm1p binding sequences fused to a *LacZ* reporter (2xRLM1-*LacZ*), were grown to the exponential phase and the β-galactosidase activity was measured as described in *Experimental Procedures*. Values are mean ± SD

of at least three independent experiments. ****p<0.0001; ***p<0.001; *p<0.05. **C.** Hog1p activation was monitored in BY4741, *isc1*Δ*, sit4*Δ and *isc1*Δ*sit4*Δ cells by immunoblotting, as described in A. A representative blot out of three is shown. **D.** Hog1p activation was monitored in BY4741, *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells by immunoblotting, as described in A (left panel). BY4741 and *sch9*Δ cells were grown to the exponential phase and treated with either 10 μM C_2-ceramide or DMSO (vehicle) for 1 h (right panel). A representative blot out of three is shown.

It was previously shown that Hog1p activation leads to its import into the nucleus where it phosphorylates the Msn2p/Msn4p, Hot1p, Sko1p and Smp1p transcription factors to promote adaptation to stress conditions (Marquez *et al.*, 1998, Reiser *et al.*, 1999, Rep *et al.*, 1999, Rep *et al.*, 2000, Alepuz *et al.*, 2001, Proft *et al.*, 2001, de Nadal *et al.*, 2003, de Nadal *et al.*, 2004, O'Rourke & Herskowitz, 2004, Proft *et al.*, 2006). Thus, we have also monitored Hog1p cell localization by fluorescence microscopy in cells expressing *HOG1-GFP* (Figure 2.S4). Similarly to parental cells, Hog1p was present in the cytoplasm of *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ mutants, implying that TORC1 may not regulate Hog1p localization. We have also evaluated the activation of the Cell Wall Integrity (CWI) pathway, since we have previously reported a Hog1p-dependent activation of Slt2p, a MAPK of the CWI pathway in *isc1*Δ cells (Barbosa *et al.*, 2012). For this purpose, we monitored the activation of Rlm1p, a transcription factor regulated by Slt2p, by measuring β-galactosidase activity in cells expressing a LacZ reporter under the control of Rlm1p promoter. Consistent with the hyperactivation of Hog1p and induction of the Rlm1p-driven LacZ reporter, β-galactosidase activity was increased by 1.7- and 4.3-fold in *isc1*Δ and *isc1*Δ*tor1*Δ cells, respectively (Figure 2.6.B).

It was previously shown that the protein phosphatase Sit4p is negatively regulated by TORC1 signalling (Jacinto *et al.*, 2001) but activated by ceramide (Nickels & Broach, 1996). Moreover, the deletion of *SIT4* suppresses *isc1*Δ phenotypes (Barbosa *et al.*, 2011). Notably, Hog1p phosphorylation increased in *sit4*Δ cells and it was exacerbated in *isc1*Δ*sit4*Δ cells (Figure 2.6.C), as observed in *isc1*Δ*tor1*Δ cells (Figure 2.6.A). This suggests that the phosphorylation of Hog1p is also regulated by a Sit4p-dependent mechanism. Remarkably, the deletion of *SCH9* decreased Hog1p phosphorylation in *isc1*Δ cells (Figure 2.6.D). It was

previously reported that ceramide signalling increases Hog1p phosphorylation (Barbosa *et al.*, 2012). Thus, we tested if Sch9p regulates the HOG pathway in response to ceramide. As previously reported, C_2-ceramide treatment increased Hog1p phosphorylation in parental cells (Figure 2.6.D). In contrast, the deletion of *SCH9* completely abolished Hog1p phosphorylation upon treatment with ceramide (Figure 2.6.D). These results suggest that Sch9p is acting upstream of Hog1p in response to ceramide signalling.

Overall, the results show that the deletion of *TOR1* or *SIT4* does not suppress *isc1*Δ phenotypes through the attenuation of Hog1p hyperactivation and Sch9p appears to regulate the activation of the HOG pathway in response to ceramide in Isc1p-deficient cells.

2.3. Discussion

The TORC1 pathway is a well-established nutrient response pathway that modulates aging and age-related diseases (Johnson *et al.*, 2013). Here we provided evidence that TORC1 signalling is deregulated in cells lacking Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase. Isc1p-deficient cells exhibit increased TORC1 activity, which is detrimental for this mutant. In fact, the deletion of *TOR1* alleviates the premature aging, oxidative stress sensitivity and mitochondrial dysfunctions of *isc1*Δ cells. However, *isc1*Δ*tor1*Δ cells exhibit lower resistance to oxidative stress, shortened CLS, slightly impaired mitochondrial function, and higher levels of ROS and apoptotic cell death markers compared to the *tor1*Δ mutant strain, suggesting that TORC1-independent mechanisms also contribute to *isc1*Δ phenotypes. In agreement, the overexpression of *ISC1* does not suppress the rapamycin hypersensitivity of *tor1*Δ mutants, suggesting that Isc1p is not acting downstream of TORC1 (Figure 2.S5).

Several lines of evidence suggest an intricate interplay between sphingolipid metabolism and TOR signalling. Both TORC1 and TORC2 control the biosynthesis of sphingolipids through regulation of Orm1p and Orm2p, two evolutionarily conserved integral membrane proteins of the endoplasmic reticulum (Breslow *et al.*, 2010, Liu *et al.*, 2012, Shimobayashi *et al.*, 2013). However, TORC1 and TORC2 signalling seems to function independently in the regulation of

sphingolipid metabolism. TORC1 negatively controls the synthesis of complex sphingolipids from ceramide via inhibition of Orm1/2p phosphorylation in a process mediated by Npr1p (Shimobayashi *et al.*, 2013). TORC2 stimulates the *de novo* biosynthesis of sphingolipids via activation of Ypk1p, which then phosphorylates and inactivates Orm1/2p (Roelants *et al.*, 2011). Since Orm1/2p bind to and inhibit serine palmitoyl-coenzyme A transferase (SPT) (Breslow *et al.*, 2010), which catalyzes the first step in sphingolipid biosynthesis (Yard *et al.*, 2007), its inactivation by a Ypk1p-dependent phosphorylation alleviates SPT inhibition and increases sphingolipid production. In addition, TORC2 stimulates ceramide biosynthesis by an Ypk2p-dependent mechanism (Aronova *et al.*, 2008) and inhibits Isc1p activity via modulation of Slm1/2p (Tabuchi *et al.*, 2006, Niles & Powers, 2012).

Our data suggests a genetic interaction between *ISC1* and *TOR1*. A recent report suggests a link between ceramide generated by mammalian acid sphingomyelinase under amino acid deprivation conditions and mTOR inactivation by a PP1/PP2A-dependent mechanism (Taniguchi *et al.*, 2012). Notably, yeast Isc1p appears to occupy a central position and may possibly act as a metabolic hub (together with ceramide synthase) in the TOR pathway to regulate ceramide dynamics, since Isc1p is functionally linked with both TORC1 and TORC2 branches. In particular, the modulation of complex sphingolipids turnover by coupling TORC1-regulated biosynthesis with Isc1p-driven hydrolysis may be important to control ceramide flux in yeast.

The molecular mechanism by which TORC1 is activated in *isc1*Δ cells remains to be elucidated. Nevertheless, the activation of TORC1 in Isc1p-deficient cells may be part of a homeostatic response that aims to decrease the synthesis of complex sphingolipids, since *isc1*Δ cells accumulate inositolphosphorylceramide (IPC) and mannosyldiinositolphosphorylceramide ($M(IP)_2C$) (Sawai *et al.*, 2000, Tabuchi *et al.*, 2006) and TORC1 negatively controls their biosynthesis (Shimobayashi *et al.*, 2013). However, TORC1 activation impairs mitochondrial function and oxidative stress resistance in *isc1*Δ cells, and *TOR1* deletion suppresses *isc1*Δ phenotypes. Such features are in agreement with previous studies showing that *TOR1* deletion regulates yeast CLS by a cell-intrinsic mechanism (Bonawitz *et al.*, 2007, Pan *et al.*, 2011). Reduced TORC1 signalling increases mitochondrial coupling during active growth in yeast cells, eliciting an

adaptive response that preconditions yeast cells to better survive in the stationary phase and promotes longevity (Pan *et al.*, 2011). Furthermore, *TOR1* disruption leads to an increased translation of mtDNA-encoded subunits of the oxidative phosphorylation system (Bonawitz *et al.*, 2007), which is consistent with the higher oxygen consumption and COX activity observed in this study. In addition, it improves oxidative stress resistance in stationary phase (Wei *et al.*, 2009).

In the present study, we demonstrate that TORC1-driven Sch9p C-terminal phosphorylation is increased in *isc1*Δ cells and our results are consistent with TORC1 acting through Sch9p-associated mechanisms since the disruption of both *TOR1* and *SCH9* abolishes *isc1*Δ phenotypes. Importantly, Isc1p-deficient cells presented reduced autophagic flux, both upon treatment with rapamycin and during cell aging, which is consistent with the activation of the TORC1-Sch9p pathway. In agreement, *TOR1* and *SCH9* disruption reestablished the functional integrity of autophagy in *isc1*Δ cells and this is correlated with the restoration of mitochondrial function in the double mutants, ultimately abolishing the H_2O_2 hypersensitivity and premature aging exhibited by this mutant strain.

It was previously demonstrated that the accumulation of very long chain ceramide species (dihydro-C_{26}-ceramide and phyto-C_{26}-ceramide species) and ceramide-activation of the protein phosphatase Sit4p are also implicated in *isc1*Δ phenotypes (Barbosa *et al.*, 2011). Since TORC1 negatively regulates Sit4p by promoting its association with the inhibitor Tap42p (Jacinto *et al.*, 2001) and both *TOR1* and *SIT4* deletions suppress *isc1*Δ phenotypes, we propose that Sit4p may act in parallel to TORC1 signalling, by directly sensing ceramide signals (Figure 2.7).

Several pieces of evidence suggest a functional link between sphingolipids, TOR signalling and the HOG pathway. The constitutive activation of Hog1p is deleterious for *isc1*Δ cells since the deletion of *HOG1* attenuates the shortened CLS, hydrogen peroxide sensitivity and mitochondrial dysfunctions of this mutant strain (Barbosa *et al.*, 2012). The HOG pathway is activated upon treatment of yeast cells with myriocin, an inhibitor of the *de novo* sphingolipid biosynthesis, and in cells with impaired synthesis of IPC (Tanigawa *et al.*, 2012). In addition, reduced TOR signalling lowers the basal activity of Hog1p in *Candida albicans* through the Hog1p tyrosine phosphatases Ptp2p and Ptp3p (Su *et al.*,

2013) and our results show a decrease of Hog1p basal phosphorylation in the *tor1*Δ mutant.

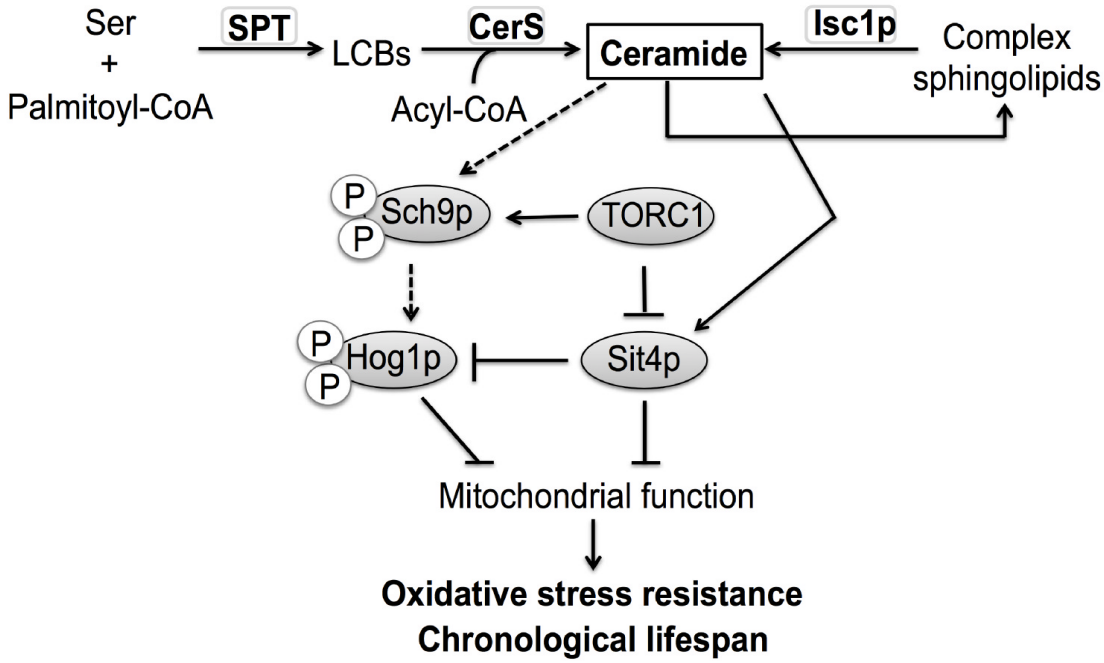


Figure 2.7. Sit4p and Sch9p integrate nutrient and stress signalling from TORC1 with ceramide signals to regulate mitochondrial function and CLS in yeast. Cells lacking Isc1p present increased TORC1-Sch9p activity, ultimately leading to mitochondrial dysfunction, decreased oxidative stress resistance and shortened CLS. Apart from sensing signals from TORC1, the ceramide activated protein phosphatase Sit4p and the AGC protein kinase Sch9p also integrate ceramide signalling derived from Isc1p to regulate mitochondrial function, oxidative stress resistance and chronological lifespan in yeast.

In this study, we demonstrate that *TOR1* deletion does not suppress Hog1p hyperactivation or alter Hog1p localization in *isc1*Δ cells. However, our results support a more intricate interplay between the two signalling pathways in *isc1*Δ cells. Indeed, Hog1p phosphorylation was even increased in *isc1*Δ*tor1*Δ cells in comparison to *isc1*Δ cells and remarkably a similar feature was also observed for both *sit*4Δ and *isc1*Δ*sit*4Δ cells, which present extended lifespan when compared to *isc1*Δ or even to parental cells (Barbosa *et al.*, 2011). We propose that the deletion of *TOR1* or *SIT4* either suppresses putative deleterious effects of Hog1p hyperactivation on mitochondrial function and CLS or modulates the regulatory role of Hog1p to determine cell fate in Isc1p-deficient cells. In fact, the activation of

yeast Hog1p or its mammalian orthologue, p38 MAPK, has been implicated in both cell survival and cell death. For example, *HOG1* deletion decreases osmotic and oxidative stress resistance and shortens CLS in yeast (Kaeberlein *et al.*, 2002, Bilsland *et al.*, 2004). However, the expression of constitutively active Hog1p mutant kinases or constitutive activation of the HOG pathway is lethal (Maeda *et al.*, 1993, Maeda *et al.*, 1994, Wurgler-Murphy *et al.*, 1997), and hyperactivation of Hog1p is detrimental under heat stress conditions (Lee *et al.*, 2002). In mammalian cells, p38 MAPK is activated during the onset of senescence (Wang *et al.*, 2002, Deng *et al.*, 2004), its inhibition moderately delays replicative senescence (Iwasa *et al.*, 2003) and its activation promotes apoptosis (Xia *et al.*, 1995, Grethe *et al.*, 2004).

Remarkably, we provide significant evidence that the activation of the *HOG* pathway is modulated by ceramide signalling by Sch9p-dependent mechanisms, ultimately contributing to *isc1*Δ phenotypes. Indeed, C_2-ceramide-induced Hog1p phosphorylation (Barbosa *et al.*, 2012) was suppressed by the deletion of *SCH9*. In addition, Hog1p phosphorylation is decreased upon deletion of *SCH9* in Isc1p-deficient cells and the results are consistent with both proteins acting in the same pathway since *SCH9* (this study) and *HOG1* (Barbosa *et al.*, 2012) deletions suppress *isc1*Δ phenotypes. Therefore, our study points out to a model in which Sch9p integrates nutrient and stress signals from TORC1 with ceramide signalling, the latter contributing to the modulation of the Hog1p phosphorylation (Figure 2.7). Interestingly, this appears to be ceramide-specific since the addition of PHS had no effect on Hog1p phosphorylation (data not shown). It is known that Sch9p possesses a C_2-domain, which is known to bind to several substrates, namely Ca^{2+}, phospholipids, inositol polyphosphates, and intracellular proteins (Nalefski & Falke, 1996). Whether ceramide acts by binding directly to the C_2-domain remains to be established.

Importantly, we also demonstrate that Isc1p-deficient cells presented reduced autophagic flux, which could further contribute to mitochondrial dysfunction. Remarkably, *TOR1* and *SCH9* disruption reestablishes the functional integrity of autophagy in *isc1*Δ cells, which is consistent with the restoration of mitochondrial function in the double mutants, possibly abolishing the H_2O_2 hypersensitivity and premature aging exhibited by this mutant strain.

In summary, our data implicate TORC1-Sch9p activation in the mitochondrial dysfunction, premature aging, oxidative stress sensitivity and impaired autophagy exhibited by *isc1*Δ cells. However, Isc1p and TORC1 also seem to act through independent pathways, as *isc1*Δ*tor1*Δ phenotypes are intermediate to those displayed by *isc1*Δ and *tor1*Δ cells. The lifespan extension in *isc1*Δ cells imparted by reduced TORC1 signalling is not associated with the attenuation of Hog1p hyperphosphorylation. Our results suggest that complex signalling interconnections involving TORC1 and its downstream effectors Sch9p and Sit4p govern redox homeostasis and lifespan of *isc1*Δ cells and support a model in which both proteins act as physiological core centers integrating nutrient and stress signal from TORC1 and ceramide signalling derived from Isc1p to regulate mitochondrial function and CLS in yeast (Figure 2.7).

2.4. Experimental procedures

2.4.1. Yeast cells and growth conditions

S. cerevisiae BY4741 was the parental strain of all haploid derivatives used in this study (Table 2.5.1). Yeast cells were grown aerobically at 26°C in a gyratory shaker (at 140 r.p.m.), with a ratio of flask volume/ medium volume of 5:1. The growth media used were YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose], YPGlycerol [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 4% (v/v) glycerol] or synthetic complete (SC) drop-out medium containing 2% (w/v) glucose, 0.67% yeast nitrogen base without amino acids and supplemented with appropriate amino acids (80 mg histidine L^{-1}, 400 mg leucine L^{-1}, 80 mg tryptophan L^{-1} and 80 mg uracil L^{-1}). The *tor1*Δ cells were obtained by replacing *TOR1* with a *tor1*::*KanMX4* deletion cassette amplified by polymerase chain reaction (PCR) using the corresponding strain available from EUROSCARF (Germany) and the following primers: Fw (GAGAATCATTACCGGCGAAA) and Rv (ACGAACACGTTTTGGTGATG). For *ISC1* disruption in *tor1*Δ and *sch9*Δ::*KanMX4* cells, a deletion fragment containing *LEU2* and the flanking regions of *ISC1* was amplified by PCR using pRS315 and primers Fw

(ATTTGCGCTTTCCGTAAAAAGGGAAAAAAAGCAGATATTTAAGCAAGGATTTTCT) and Rv (TCAGTAATTTTTTTACATATGCTAAAGAAAATCGATAATACCGCATATCGACCCTCGAGGAG). Cells were transformed by electroporation. The *tor1*Δ cells were selected in YPD medium containing geneticin (0.4 $mg.mL^{-1}$) whereas *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells were selected in minimal medium lacking leucine [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose supplemented with appropriate amino acids (40 mg histidine L^{-1}, 40 mg uracil L^{-1} and 40 mg methionine L^{-1})]. The correct insertion of all marker cassettes was confirmed by PCR.

To evaluate TORC1-dependent C-terminal phosphorylation of Sch9p, BY4741 and *isc1*Δ cells were transformed by electroporation with pJU676 (Urban *et al.*, 2007) and selected in minimal medium lacking uracil. For the analysis of mitochondrial morphology, yeast cells were transformed with a plasmid expressing mitochondrial DsRed (pYX222-mtDsRed) and selected in minimal medium lacking histidine. To evaluate autophagic flux, cells were transformed by electroporation with pRS416-GFP-*ATG8* (Yorimitsu *et al.*, 2007) and selected in minimal medium lacking uracil. For Hog1p localization assays, yeast cells were transformed with pRS416-*HOG1*-GFP (Ferrigno *et al.*, 1998) and selected in minimal medium lacking uracil. To assess the activation of the CWI pathway by Rlm1p reporter transcriptional activity, yeast cells were transformed with pLGΔ312-2xRLM1-*LacZ* (Jung *et al.*, 2002) and selected in minimal medium lacking uracil. For epistatic analysis, BY4741 and *tor1*Δ strains were transformed with pYES2 and pYES2-*ISC1* (Almeida *et al.*, 2008) and selected in minimal medium lacking uracil.

2.4.2. Stress resistance and chronological lifespan

For H_2O_2 resistance assay, cells were grown to the exponential phase (OD_{600} = 0.6) or early stationary phase (48h after exponential phase) and exposed to 1.5 mM or 300 mM H_2O_2 (Merck) for 60 and 30 min, respectively. The CLS assay was performed as described (Fabrizio & Longo, 2003). For both assays, cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % (w/v) agar and expressed as the percentage of the colony-forming units after growth at 26ºC for 3 days (time *T* vs. T_0 (when viability was

considered 100%) for the CLS assay; treated vs. untreated cells for H_2O_2 resistance). Values are mean ± SD of at least three independent experiments.

2.4.3. Enzymatic activities and oxygen consumption

For enzyme activities, yeast cells were harvested by centrifugation for 5 min at 4,000 r.p.m. (4ºC). Cells were then ressuspended in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein) and phosphatase inhibitors (50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate). Total protein extracts were obtained by mechanical disruption through vigorous shaking of the cell suspension in the presence of glass beads for 5 min. Short pulses of 1 min were applied followed by 1 min incubation on ice. Cell debris was removed by centrifugation at 13,000 r.p.m. for 15 min and protein content was determined by the method of Lowry, using bovine serum albumin as a standard. Catalase activity was analyzed *in situ,* in the presence of 3,3′-diaminobenzidine tetrahydrochloride, using the H_2O_2/peroxidase system (Conyers & Kidwell, 1991). Cytochrome *c* oxidase (COX) activity was determined by measuring cytochrome *c* oxidation (Poyton *et al.*, 1995). β-galactosidase activity determination was performed as previously reported (Barbosa *et al.*, 2012). Oxygen consumption rate was measured for 3 x 10^8 cells in PBS buffer (pH 7.4), using an oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software.

2.4.4. Mitochondrial membrane potential, ROS levels and cell death

The mitochondrial membrane potential was measured using the potential-sensitive dye $DiOC_6(3)$. Briefly, 2x10^6 cells were ressuspended in sample buffer [10 mM 2-(N-morpholino) ethanesulfonic acid, 0.1 mM $MgCl_2$ and 2% (w/v) glucose, pH 6.0]. $DiOC_6(3)$ (Molecular Probes) was added to a final concentration of 1 nM. The cell suspension was then incubated for 30 min at 26°C, collected by centrifugation and washed twice with PBS. Fluorescence was measured on the FL-1 channel of a Becton Dickinson FACS Calibur Analytic Flow cytometer with excitation and emission settings of 488 nm and 515–545 nm, respectively, without

compensation. For the quantification of ROS levels, $5x10^6$ cells were ressuspended in PBS and the superoxide anion sensitive probe dihydroethidium (DHE, Molecular Probes) was added to a final concentration of 5 µM. Cells were incubated for 10 min at 26°C, pelleted by centrifugation, washed twice with PBS and analyzed by flow cytometry with excitation and emission settings of 488 nm and ≥670 nm (FL-3 channel), without compensation.

For the characterization of the cell death process, cells were dually stained with $DiOC_6(3)$ (1 nM) and propidium iodide (PI, 2 µg/mL, Molecular Probes) to evaluate mitochondrial membrane polarization and the plasma membrane integrity, respectively. Cells were incubated for 30 min at 26°C and harvested as previously described. After suitable compensation, fluorescence was measured by flow cytometry at different wavelengths: excitation/emission at 488/525 nm for $DiOC_6(3)$ (FL-1 channel), and at 536/600 nm for PI (FL-3 channel). Data treatment was performed using the FlowJo software (Tree Star).

2.4.5. Western Blot analysis

To evaluate the TORC1-dependent C-terminal phosphorylation of Sch9p, cells transformed with pJU676 (expressing *SCH9-5HA*) were grown in SC-medium lacking uracil to the exponential phase. NTCB-chemical fragmentation analysis was done as described (Urban *et al.*, 2007). Proteins were then analyzed by SDS-PAGE using 10% polyacrylamide gels and blotted onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, United Kingdom). Immunodetection was performed using rabbit anti-HA (Sigma-Aldrich) at a 1:1,000 dilution as primary antibody, goat anti-rabbit IgG-peroxidase (Sigma-Aldrich) at a 1:5,000 dilution as secondary antibody, and the Lumigen HRP chemiluminescent substrate (GE Healthcare, RPN2109, Buckinghamshire, United Kingdom).

To monitor Hog1p phosphorylation, yeast cells were grown to the exponential phase and protein extracts (50 µg) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was incubated with the primary antibodies rabbit anti-phospho-p38 MAPK (Cell Signalling Technology, Denver, USA) at a 1:500 dilution or mouse anti-Pgk1p (Invitrogen, Carlsbad, USA) at a 1:30,000 dilution. Subsequently, the membrane was incubated with the secondary antibodies, anti-rabbit IgG-peroxidase (Sigma-Aldrich) at a 1:5,000

dilution, or anti-mouse IgG-peroxidase (Molecular Probes) at a 1:3,000 dilution. Immunodetection was performed as described above.

The evaluation of LCBs mediated Phk1/2p-dependent phosphorylation of Sch9p was performed as described (Huang *et al.*, 2012). To assess alterations in autophagic flux, cells were grown to the exponential phase in SC-medium and treated with either rapamycin (200 ng/mL, (Sigma-Aldrich) or DMSO (vehicle, Sigma-Aldrich) for 3 hours. For CLS assay in water, cells were grown to the PDS phase, washed twice with water and then maintained in water overtime. Total protein extracts (30 µg) were analyzed in similar conditions as previously described. The membrane was incubated with the primary antibodies mouse anti-GFP (Roche, Basel, Switzerland) at a 1:3,000 dilution or mouse anti-Pgk1p (Invitrogen, Carlsbad, USA) at a 1:30,000 dilution. Subsequently, the membrane was incubated with the secondary antibody anti-mouse IgG-peroxidase (Molecular Probes) at a 1:3,000 dilution. Immunodetection was done as described.

2.4.6. Fluorescence microscopy

For mitochondrial morphology analysis, cells transformed with pYX222-mtDsRed were grown in SC-medium lacking histidine to the exponential or PDS phase. To assess Hog1p cell localization, cells expressing *HOG1-GFP* were grown in SC-medium lacking uracil to the exponential phase and stained with the fluorescent dye 4',6'-diamidino-2-phenylindole, DAPI (Molecular Probes, Invitrogen, 2.5 µg/mL) to label the nucleus. Live cells were observed by fluorescence microscopy (AxioImager Z1, Carl Zeiss). Data image stacks were deconvolved by QMLE algorithm of Huygens Professional v3.0.2p1 (Scientific Volume Imaging B.V.). Maximum intensity projection was used to output final images using ImageJ 1.45v software.

2.4.7. Statistical analysis

Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's *t-test.* The 0.05 probability level was chosen as the point of statistical significance throughout. Statistical analyses were carried out using GraphPad Prism Software v5.01 (GraphPad Software).

Acknowledgements

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2.5. Supplementary Information

Table 2.5.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4741	Mata *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* [pJU676, pYX222-mtDsRed, pRS416-*GFP-ATG8*, pRS416-*HOG1-GFP,* pLGΔ312-2xRLM1-*LacZ,* pYES2, pYES2- *ISC1*]	EUROSCARF
*isc1*Δ	BY4741 *isc1*Δ::*KanMX4* [pJU676, pYX222-mtDsRed, pRS416-*GFP-ATG8*, pRS416-*HOG1-GFP,* pLGΔ312-2xRLM1-*LacZ*]	EUROSCARF
*tor1*Δ	BY4741 *tor1*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*, pRS416-*HOG1-GFP,* pLGΔ312-2xRLM1-*LacZ,* pYES2, pYES2-*ISC1*]	This study
*isc1*Δ*tor1*Δ	BY4741 *isc1*Δ::*LEU2 tor1*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*, pRS416-*HOG1-GFP,* pLGΔ312-2xRLM1-*LacZ*]	This study
*sch9*Δ	BY4741 *sch9*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*]	EUROSCARF
*isc1*Δ*sch9*Δ	BY4741 *isc1*Δ::*LEU2 sch9*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*]	This study
*sit4*Δ	BY4741 *sit4*Δ::*KanMX4*	EUROSCARF
*isc1*Δ*sit4*Δ	BY4741 *isc1*Δ::*URA3 sit4*Δ::*KanMX4*	Barbosa *et al.* (2011)

Harboring plasmids are shown in square brackets

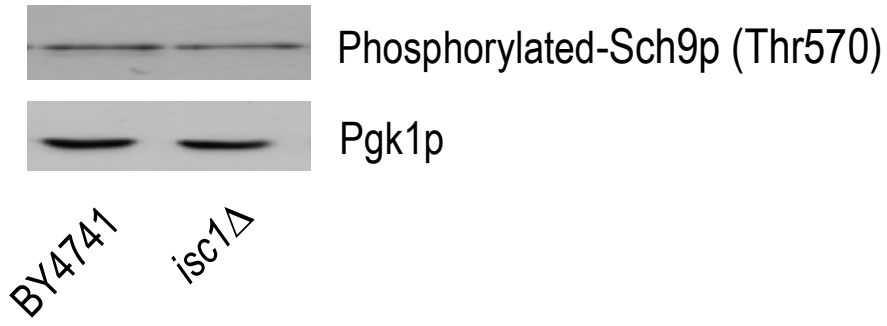

Figure 2.S1. The Pkh1/2p-dependent Sch9p phosphorylation does not contribute to *isc1*Δ phenotypes. The Pkh1/2p-mediated phosphorylation of Sch9p at Thr570 was monitored in BY4741 and *isc1*Δ cells by immunoblotting, using anti-phospho-Thr570-Sch9p antibody (top panel) or anti-Pgk1p (loading control) as primary antibodies. A representative blot is shown.

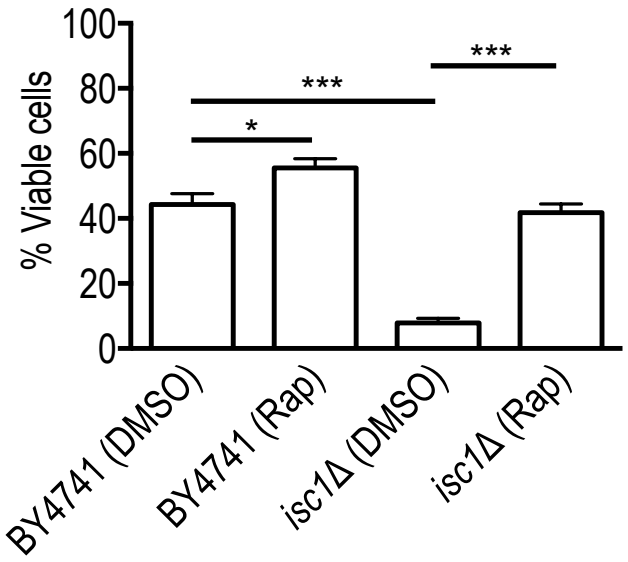

Figure 2.S2. **Inhibition of TORC1 signalling with rapamycin suppresses oxidative stress sensitivity displayed by *isc1*Δ cells.** Yeast cells were grown in SC-medium to the early exponential phase (OD_{600}=0.3), pre-incubated with either 200 ng/mL rapamycin (Rap) or vehicle (DMSO) for 3h and then treated with 1.5 mM H_2O_2 for 60 min. Cell viability was determined as described in *Experimental Procedures*. ***p<0.001; *p<0.05.

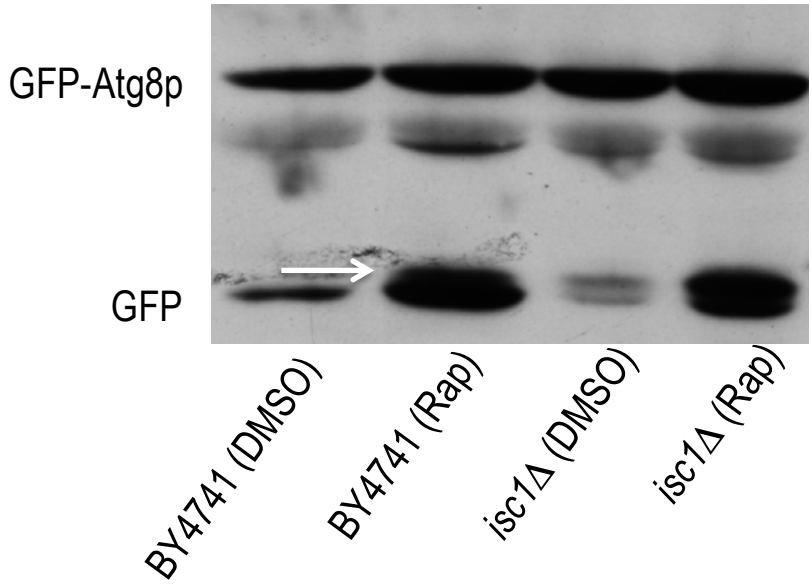

Figure 2.S3. **The autophagic flux is decreased in *isc1*Δ cells.** *S. cerevisiae* BY4741 and *isc1*Δ cells carrying pRS416 *GFP-ATG8* were grown to the exponential phase in SC-medium and treated with either 200 ng/mL rapamycin (Rap) or DMSO (vehicle) for 3 hours. Proteins were analyzed by immunoblotting, using anti-GFP antibody. The Western blot shown here is a replicate of that shown in Figure 2.4.C (only for BY4741 and *isc1*Δ cells) with a longer exposure time. A slower migrating band (above free GFP) was observed in *isc1*Δ cells, both under basal conditions and upon rapamycin treatment. A similar upper band (marked with an arrow) was observed in parental cells upon treatment with rapamycin. Notably, this upper band was more abundant in *isc1*Δ cells when compared to the one observed in parental cells. It probably results from impaired or aberrant GFP-Atg8p processing, possibly due to vacuolar dysfunction (defective Pep4p-dependent proteolytic activity) or alterations in vacuolar morphology upon deletion of *ISC1*, as mentioned in the main text. Thus, we have considered only the higher migrating band as free GFP (generated from processing of GFP-Atg8p) for autophagic flux quantification (Fig. 4D).

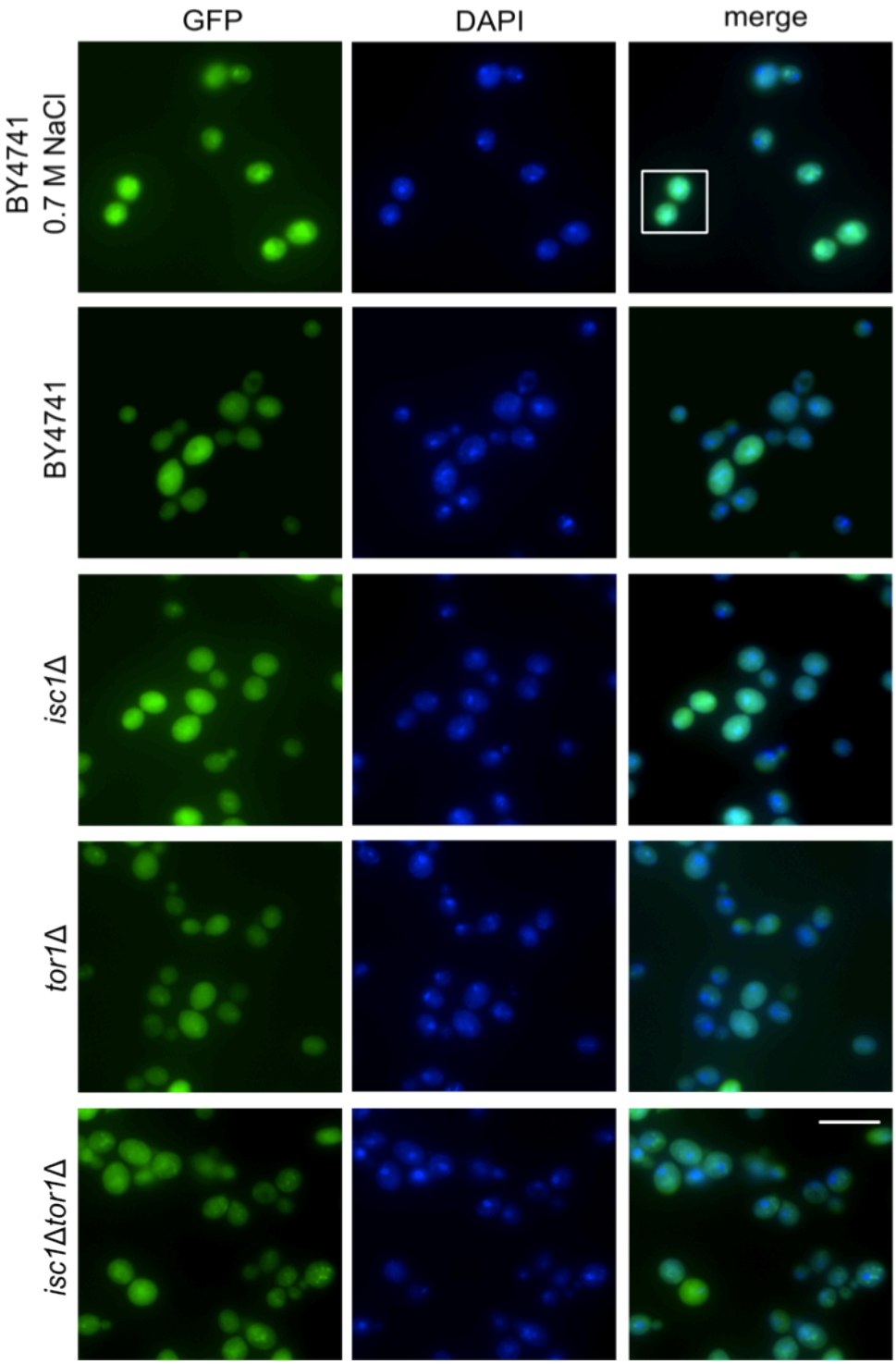

Figure 2.S4. The deletion of *TOR1* does not alter Hog1p localization in *isc1*Δ cells. *S. cerevisiae* BY4741, *isc1*Δ, *tor1*Δ and *isc1*Δ*tor1*Δ cells transformed with pRS416-*HOG1-GFP* were grown to the exponential phase and live cells were visualized by fluorescence microscopy for GFP and DAPI, as described in *Experimental Procedures*. BY4741 cells treated with 0.7 M NaCl for 20 min were used as positive control to monitor Hog1p translocation to the nucleus. Scale bar: 5 μm.

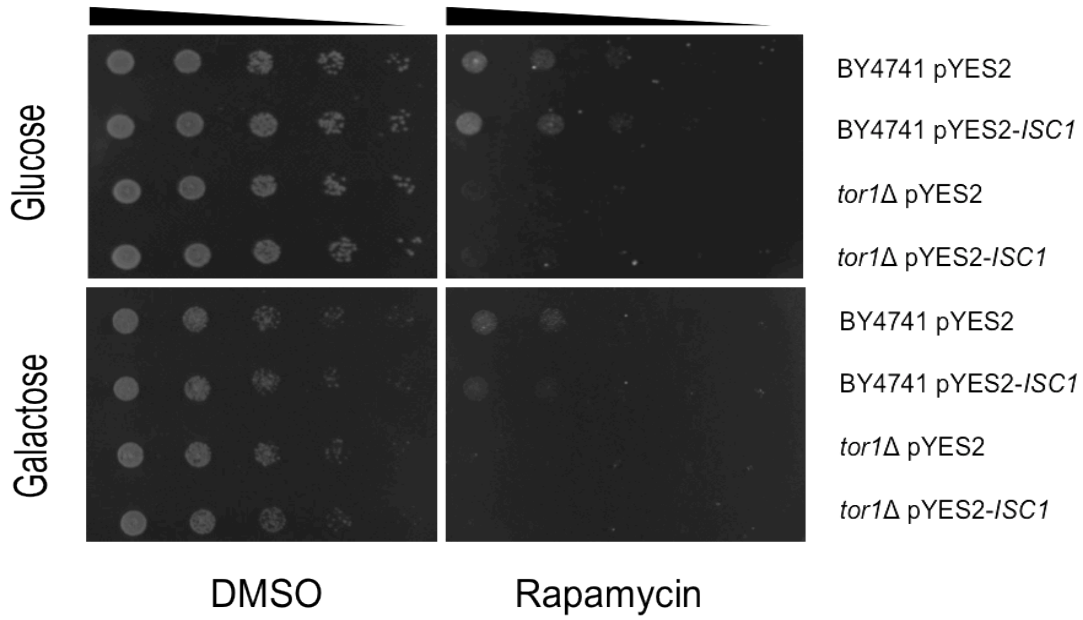

Figure 2.S5. The rapamycin sensitivity of the *tor1*Δ mutant was not suppressed by *ISC1* overexpression. *S. cerevisiae* BY4741 and *tor1*Δ cells carrying pYES2 or pYES2-*ISC1* were grown in SC-medium to the exponential phase and then diluted to OD_{600}=0.1. Fivefold dilution series were spotted on glucose or galactose media containing either rapamycin (50 ng/mL, dissolved in DMSO) or DMSO (vehicle) and cells were grown at 26°C for 5 days.

CHAPTER III

One of the major challenges for (...) who wish to work in the domain of (...) biology is becoming conversant with the daunting intricacies of existing biological knowledge. Questions about the origin, function, and structure of living systems have been pursued by nearly all cultures throughout history, and the work of the last generations has been particularly fruitful. The knowledge of living systems resulting from this research is far too detailed and complex for any one human to comprehend.

Lawrence Hunter

Isc1p-driven ceramide signalling regulates vesicular trafficking, mitophagy and mitochondrial dynamics in yeast

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Keywords: Aging; ceramide; Dnm1p; Isc1p; macroautophagy; mitochondrial dynamics; mitochondrial function; mitophagy

Abstract

Macroautophagy plays an important regulatory role in mitochondrial function, stress response and aging. It is deregulated in yeast cells lacking Isc1p, an orthologue of mammalian neutral sphingomyelinase type 2, but the impact of sphingolipid metabolism on autophagy still remains largely unknown. Here, we show that Isc1p deficient cells present reduced autophagic flux due to impaired vesicular trafficking and vacuolar function (protein sorting, proteolysis and acidification) and to vacuolar fragmentation. These phenotypes are suppressed by downregulation of TORC1 or its downstream effectors, the AGC protein kinase Sch9p and the PP2A-like phosphatase Sit4p, which integrate nutrient and stress signals from TORC1 with ceramide signalling from Isc1p. Notably, *isc1*Δ cells display hyperactivation of mitophagy and higher levels of Dnm1p associated with unbalanced mitochondrial fission, leading to mitochondrial fragmentation, oxidative stress sensitivity and shortened lifespan. Moreover, Isc1p and Dnm1p physically interacted *in vitro*, further supporting a regulatory role for Isc1p on mitochondrial dynamics. Overall, our work demonstrates that Isc1p-mediated ceramide signalling regulates macroautophagy, mitophagy and mitochondrial dynamics in yeast with impact on lifespan.

3.1. Introduction

Sphingolipids are important structural components of cell membranes found in all eukaryotes and some prokaryotic organisms (Dickson & Lester, 1999, Dickson, 2008, Hannun & Obeid, 2008, Pruett *et al.*, 2008). Sphingosine, ceramide and sphingosine-1-phosphate (S1P) are core sphingolipids regulating fundamental cellular processes including cell growth, senescence, apoptosis and autophagy (Zheng *et al.*, 2006, Dickson, 2008, Rego *et al.*, 2012, Young *et al.*, 2013). Sphingosine and ceramide exert pleiotropic effects on protein kinases and phosphatases and induce cell cycle arrest and apoptosis (Hannun & Obeid, 2008). In particular, ceramide regulates apoptosis (Obeid *et al.*, 1993, Mullen & Obeid, 2012) and cell senescence (Venable *et al.*, 1995) by modulating the activity of ceramide-activated protein kinases (e.g. PRKCD/PKC) and phosphatases (CAPP, PP1 and PP2A) (Hannun & Obeid, 2008). In contrast, S1P promotes cell proliferation and survival by acting on receptors (Hla, 2004, Hannun & Obeid, 2008).

The budding yeast *Saccharomyces cerevisiae* has been important to understand the mechanisms for sphingolipid homeostasis in mammals due to the high conservation of this metabolism (Dickson, 2008). Therefore, it is a useful model organism to study overall sphingolipid metabolism and functions (Rego *et al.*, 2014). Initial studies have demonstrated that Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase-2 (nSMase2) responsible for the hydrolysis of inositol phosphosphingolipids to produce ceramide, is implicated in oxidative stress resistance, mitochondrial function and chronological lifespan (CLS) (Almeida *et al.*, 2008). Indeed, *isc1*Δ cells display premature aging, increased hydrogen peroxide sensitivity and several mitochondrial dysfunctions (Almeida *et al.*, 2008). The ceramide-activated protein phosphatase Sit4p, related to the type 2A family of protein phosphatases and with high homology to human protein phosphatase 6 (Bastians & Ponstingl, 1996), becomes activated in response to increased specific ceramide species during aging in *isc1*Δ cells and contributes to these phenotypes (Barbosa *et al.*, 2011).

More recently, we showed that Isc1p deficiency leads to the activation of the TORC1-Sch9p and the HOG pathways, the latter in response to ceramide

signalling by Sch9p-dependent mechanisms, contributing to its phenotypes (Barbosa *et al.*, 2012, Teixeira *et al.*, 2014).

TORC1 is a major regulator of autophagy, a conserved process in eukaryotic cells involving the engulfment of cytoplasmic cargo into double-membrane vesicles named autophagosomes. After fusing to the vacuole, the content is degraded by the resident hydrolases (Reggiori & Klionsky, 2013). Several Atg proteins are organized into functional complexes that mediate these steps (Huang & Klionsky, 2002, Nakatogawa *et al.*, 2009, Boya *et al.*, 2013). Upon starvation, autophagy becomes activated, providing a source of nutrients and energy (Ryter *et al.*, 2013, Russell *et al.*, 2014). In yeast, PKA and Sch9p negatively regulate autophagy, in parallel to the TORC1 pathway, by Msn2p/Msn4p and Rim15p dependent mechanisms (Yorimitsu *et al.*, 2007). Pho85p also plays a regulatory role in this process (Budovskaya *et al.*, 2004, Yang *et al.*, 2010). Under nutrient-rich conditions, autophagy is inhibited because TORC1 is activated and hyperphosphorylates Atg13p, decreasing its affinity for Atg1p and Atg17p (Kamada *et al.*, 2010).

The view of mitochondrial dynamics has expanded into an integral cell biological process influencing cell death and aging (Berman *et al.*, 2008, Braun & Westermann, 2011). Mitochondria are dynamic structures that migrate throughout the cell, fuse and divide, and undergo regulated turnover (Westermann, 2010). The best-studied proteins involved in mitochondrial fusion are Fzo1p (the yeast homolog of mammalian mitofusins MFN1 and MFN2) and Ugo1p in the outer membrane, and Mgm1p, a dynamin-related GTPase located in the intermembrane space. The regulation of mitochondrial fission involves Fis1p, an adaptor protein in the mitochondrial outer membrane, and the cytosolic effector Dnm1p, which is recruited at sites of mitochondrial division on the surface (Westermann, 2008).

Mitochondrial dynamics and the selective degradation of mitochondria by mitophagy are important for proper mitochondrial function by allowing mitochondrial compartmentalization, mitochondrial communication and regulation of the mitochondrial shape (Detmer & Chan, 2007, Berman *et al.*, 2008, Chen & Chan, 2009, Westermann, 2012). Importantly, mitophagy is associated with the removal of dysfunctional mitochondria, therefore contributing to sustainable mitochondrial function (ATP production) (Gomes & Scorrano, 2013). Several Atg proteins are required for mitophagy (Bhatia-Kiššová & Camougrand, 2010, Kanki

& Klionsky, 2010a, Kanki *et al.*, 2010b), including Atg32p (Kanki *et al.*, 2009a) and Atg33p (Kanki *et al.*, 2010b), which are selectively involved in this process. Mitophagy and mitochondrial dynamics seem to act in a coordinated manner to assure the connectivity and maintenance of proper mitochondrial function, which are important factors determining cellular responses to the metabolic status and other factors, such as calcium and other pro-apoptotic signals (Seo *et al.*, 2010, Mao *et al.*, 2013).

In this study, we show that Isc1p is crucial for the regulation of macroautophagy, mitophagy and mitochondrial dynamics in yeast. We show that *isc1*Δ cells displayed reduced autophagic flux due to impaired vesicular trafficking and disturbed vacuolar proteolytic activity. In contrast, mitophagy is hyperactive and this was associated with mitochondrial fragmentation and decreased cell viability in *isc1*Δ cells. This was, at least in part, attributted to activation of the ceramide-activated Sit4p and the TORC1-Sch9p pathways, ultimately contributing to mitochondrial dysfunction, oxidative stress sensitivity and shortened CLS in *isc1*Δ cells. Notably, disrupted mitochondrial dynamics in the mutant strain is attributted to a slight impairment in mitochondrial fusion events and increased levels of fission protein Dnm1p but not Fis1p levels. We also demonstrated that Isc1p and Dnm1p interact *in vitro*, suggesting a regulatory role for Isc1p in mitochondrial dynamics.

3.2. Results

3.2.1. Isc1p regulates autophagy by targeting vesicular trafficking and vacuolar proteolysis

We have previously reported that *isc1*Δ cells exhibit defective autophagic flux suppressed by the deletion of *TOR1* or *SCH9* (Teixeira *et al.*, 2014). To elucidate the role of Isc1p in this process, we have analyzed the autophagy reporter GFP-Atg8p dynamics by fluorescence microscopy in cells treated with rapamycin (Figure 3.1.A). The results show that GFP-Atg8p was properly delivered into the vacuole and processed to free GFP (inside the vacuoles). In *isc1*Δ mutants, a preferential accumulation of GFP-Atg8p was observed in the

cytosol (*circa* 90% of the analysed population). Although not apparent, we still observed GFP signal inside the vacuoles, which is consistent with the generation of free GFP derived from the processing of GFP-Atg8p (Teixeira *et al*., 2014). The localization of the phagophore assembly site (PAS) at the rim of the vacuole (visible as single green dots) was not altered in the mutant. Importantly, *isc1*Δ cells showed vacuolar fragmentation (Figure 3.1.A), suggesting that vacuolar dysfunction may contribute to the observed autophagic defects.

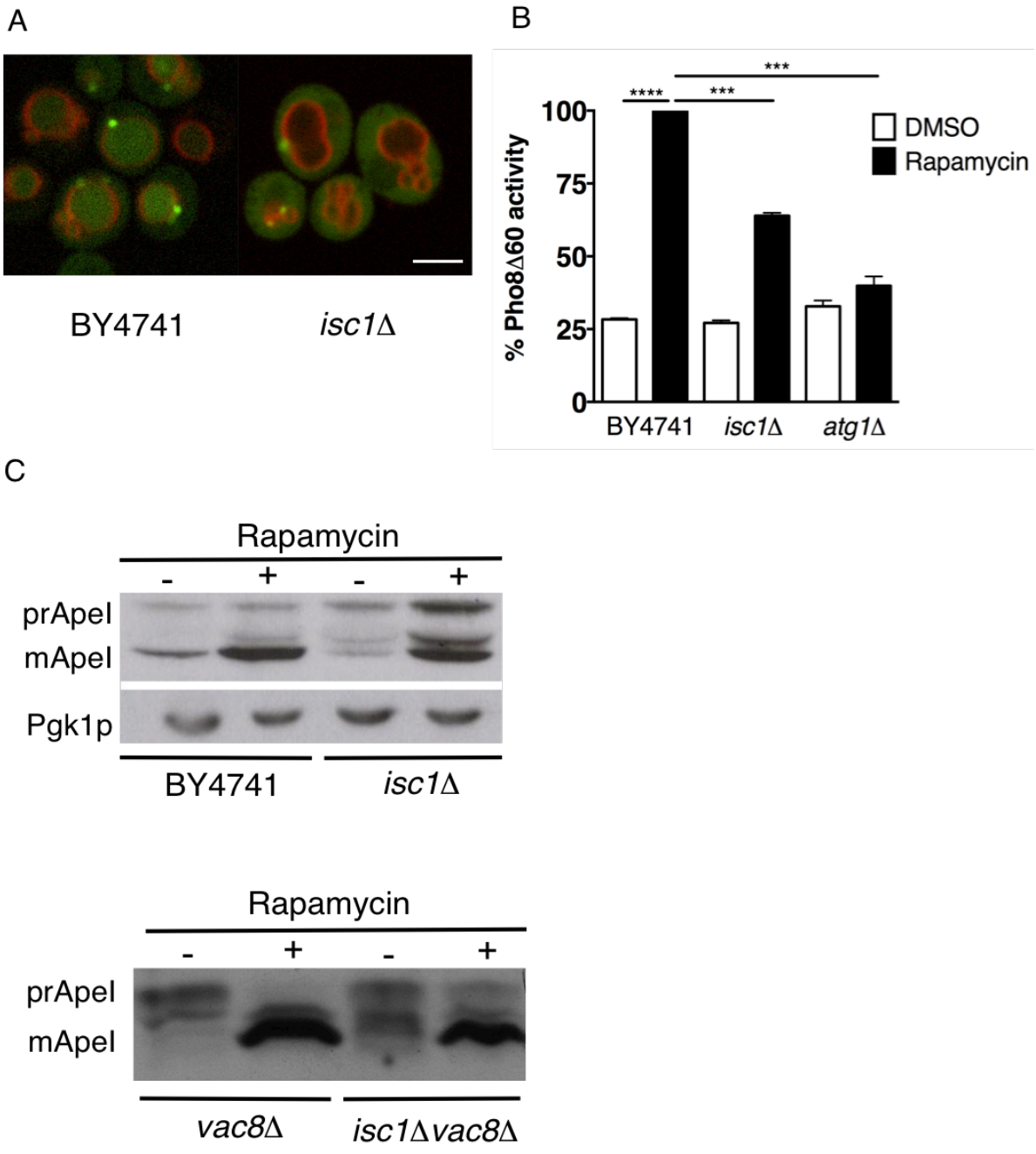

Figure 3.1. Isc1p-deficient cells exhibit vacuolar fragmentation and impaired autophagic flux.

A. BY4741 and *isc1*Δ cells expressing GFP-Atg8p were grown in SC glucose-medium to exponential phase and exposed to rapamycin (200 ng/mL) for 3h. Vacuoles were visualized by FM 4-64 staining (red) and GFP-Atg8p dynamics was observed by fluorescence microscopy. Scale bar: 5 μm. **B.** BY4741 and *isc1*Δ cells expressing Pho8Δ60 were grown in YPD to exponential phase and treated as described in A. The Pho8Δ60 activity was normalized to the activity of WT cells treated with rapamycin, which was set to 100%. Values are mean ± SD of at least three independent experiments. ****p<0.0001; ***p<0.001. **C.** The processing of the Cvt pathway marker protein prApeI to mApeI was analyzed by immunoblotting in BY4741, *isc1*Δ, *vac8*Δ and *isc1*Δ*vac8*Δ cells grown to exponential phase and treated as described in A.

Next, we corroborated these results in a different strain background (W303) by measuring the activity of a truncated form of alkaline phosphatase (Pho8Δ60), which is converted to an enzimatically active form at the vacuoles upon induction of autophagy (Yorimitsu *et al.*, 2007). ALP activity is routinelly used as a quantitative measure of autophagy induction (Yorimitsu *et al.*, 2007). Rapamycin-treated parental cells showed an increase in Pho8Δ60 activity, which was suppressed in *atg1*Δ cells where autophagy is blocked. Pho8Δ60 activity was lower in *isc1*Δ cells (Figure 3.1.B), supporting that the induction of autophagy is impaired in the mutant strain and that this phenotype is not strain specific.

The cytoplasm-to-vacuole targeting (Cvt) pathway is a vesicular transport mechanism that employs most of the macroautophagy machinery to deliver several hydrolases to the vacuole, including the percursor aminopeptidase I (prApeI) (Scott *et al.*, 1996, Wang & Klionsky, 2003). We therefore sought to determine whether *isc1*Δ cells are also defective in this process by monitoring the processing of prApeI to the mature form (mApeI). We observed that prApeI accumulated in *isc1*Δ cells upon rapamycin treatment, a feature already observed at basal conditions when compared to parental cells (Figure 3.1.C). It was also analyzed the prApeI accumulation phenotype in a *vac8*Δ background. Vac8p is required for proper induction of the Cvt pathway but not for macroautophagy (Abeliovich *et al.*, 2000). Therefore, in a *vac8*Δ background, prApeI can only be converted to its mature form when autophagy is induced. Non-treated *vac8*Δ cells

accumulated prApeI and the protein was rapidly processed to mApeI after incubation with rapamycin. In contrast, the accumulation of prApeI was still observed in rapamycin-treated *isc1*Δ*vac8*Δ cells (Figure 3.1.C). Together, these results suggest that Isc1p is required for proper regulation of macroautophagy and the Cvt pathway in yeast.

To identify the steps at which autophagy is impaired in Isc1p-deficient cells, we have firstly examined vacuolar proteolysis. Pep4p constitutes the major vacuolar protease driving the maturation of vacuolar proteases. Accordingly, *pep4*Δ cells display defective GFP-Atg8p processing when autophagy is induced (Kirisako *et al.*, 1999). To evaluate if Pep4p-dependent vacuolar proteolytic activity is impaired in *isc1*Δ cells, we monitored the maturation of carboxypeptidase Y (CPY), which occurs in a Pep4p-dependent manner (Figure 3.2.A). Most of the precursor CPY (prCPY) was converted to the mature form (mCPY) in parental cells, both under basal conditions and after incubation with rapamycin. In *isc1*Δ cells, prCPY maturation was significantly decreased, even after rapamycin treatment, suggesting that Pep4p activity is reduced in the mutant strain (Figure 3.2.A). To provide further evidence that reduced Pep4p-mediated vacuolar hydrolysis contributes to reduced autophagic flux in *isc1*Δ cells, prApeI processing (which is Pep4p-dependent) was monitored in cells overexpressing *PEP4*. Indeed, the defective maturation of prApeI in *isc1*Δ cells was alleviated by *PEP4* overexpression (Figure 3.2.B).

To determine whether Isc1p is involved in autophagosome-to-vacuole fusion, we employed a protease protection assay to evaluate the sensitivity of the prApeI to an externally added protease in rapamycin-treated *isc1*Δ cells (Krick *et al.*, 2010). After removing non-lysed cells by low speed centrifugation, we separated the total lysate (Figure 3.2.C, T, lane 1) in a 13,000 x *g* supernatant (S13, lane 2) and pellet fraction (P13, lane 3). With this method, we also used internal controls to determine the efficiency of spheroplast lysis (Pgk1p), and to verify the integrity of intracellular compartments including the vacuole (prCPY) and, by extension, autophagosomes. The resistance of the lumenal propeptide (prCPY) to trypsin in the absence of Triton X-100 (Figure 3.2.C, compare lanes 3 and 4 versus lane 5) reflects the integrity of the vacuole (and autophagosomes). In this assay, the membrane fraction (P13) was untreated (lane 3) or treated with protease alone (lane 4) or with protease and detergent (lane 5).

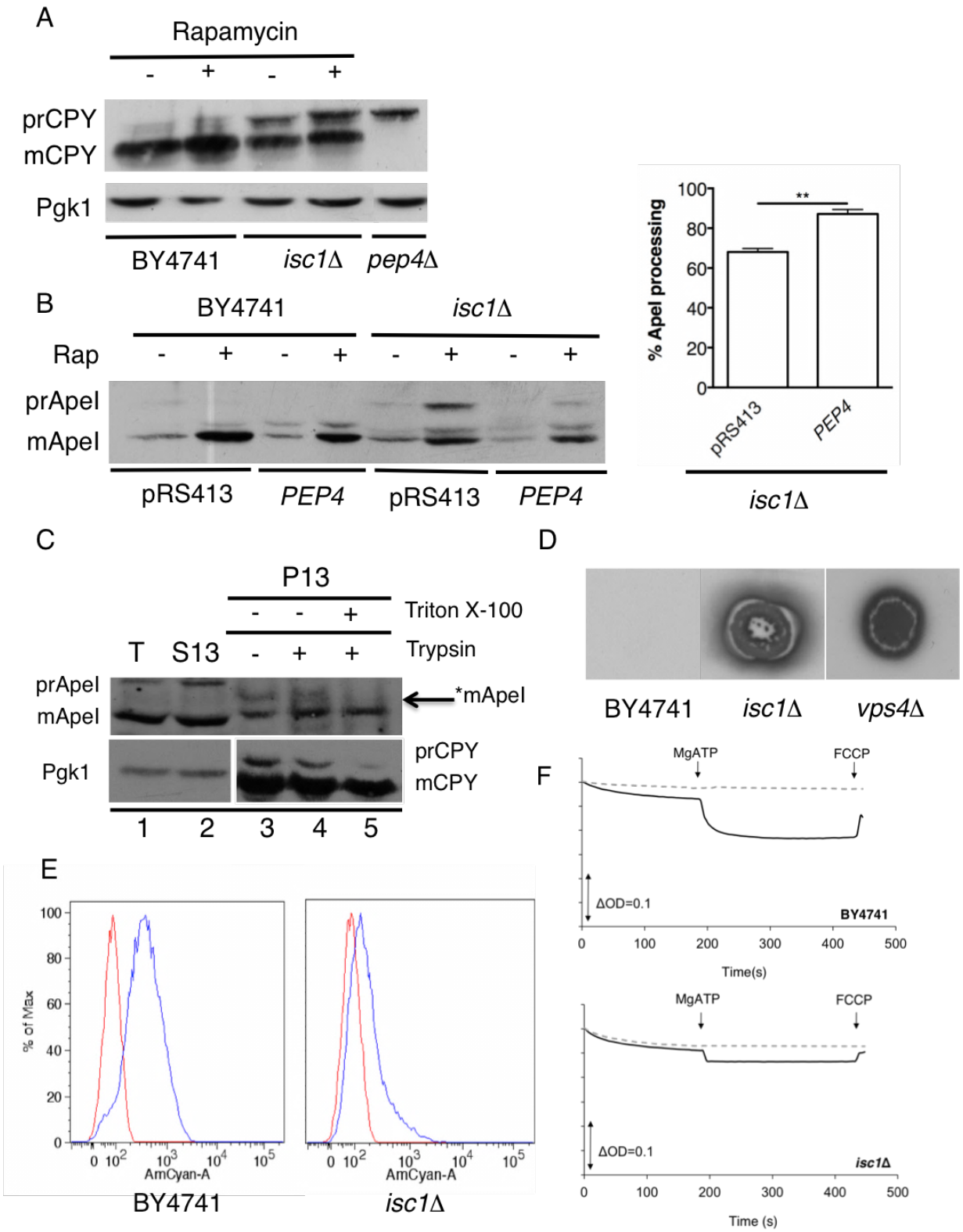

Figure 3.2. Defective vacuolar proteolysis and acidification contributed to reduced autophagic flux in *isc1*Δ cells.

A. The Pep4p-dependent processing of prCPY to mCPY was analyzed by immunoblotting in BY4741 and *isc1*Δ cells grown to exponential phase and treated with either DMSO

(vehicle) or rapamycin (200 ng/mL) for 3h. **B.** The Pep4p-dependent processing of prApeI to mApeI was analyzed by immunoblotting in BY4741 and *isc1*Δ overexpressing *PEP4* treated as described in 2A. Quantification of mApeI/(prApeI+mApeI) is shown. Values are mean ± SD of at least three independent experiments. **p<0.01. **C.** Cells devoid of Isc1p were grown to the exponential phase and treated with rapamycin (200 ng/mL) for 2h. After removing non-lysed cells, the total lysate (T, lane 1) was centirfuged to obtain a 13,000 x *g* supernatant (S13, lane 2) and pellet fraction (P13, lane 3). The P13 fraction was then ressupended in PS200 buffer (lane 3) and trypsin-digested with and without detergent (Triton X-100, lanes 4-5). The efficiency of spheroplast lysis (Pgk1p) and the integrity of intracellular compartments (vacuolar prCPY) were also monitored. *mApeI-protease-protected intermediate form of prApeI. **D.** For the detection of secreted CPY, BY4741 and *isc1*Δ cells were grown on YPD plates and overlaid with a nitrocellulose filter. The *vps4*Δ mutant was used a positive control for CPY secretion. Secreted CPY was immunodetected with anti-CPY. **E.** Vacuolar acidification of BY4741 and *isc1*Δ cells grown to exponential phase was monitored using quinacrine staining and analyzed by flow cytometry. Unprobed and probed cells are depicted in red and blue, respectively. Representative histograms are shown. **F.** Concanamycin A-sensitive vacuolar ATPase (V-ATPase) activity using acridine orange was analyzed in BY4741 and *isc1*Δ isolated vacuoles. Reactions were started in the presence of 15 μM acridine orange. When indicated, MgATP was added. Concanamycin A was added at the same time (dashed line). At the end of the assay, FCCP (15 mM) was added to dissipate the proton gradient.

If prApeI is membrane-protected (inside Cvt vesicles or within the vacuole), then prApeI is only cleaved by the exogenously added protease in the presence of detergent. Our results suggest the presence of a membrane-enclosed intermediate form of ApeI (Figure 3.2.C, compare lane 1 versus lane 3) that was fully cleaved to the mature form by trypsin in the presence of detergent (Figure 3.2.C, compare lanes 3 and 4 versus lane 5). This is consistent with our own observations that an intermediate form of ApeI accumulates in total extracts of *isc1*Δ cells, which may result from altered processing of ApeI, leading to its accumulation within the vacuoles (Figure 3.1.C). We also perfomed a similar assay using cells expressing GFP-Atg8p (Figure 3.S1). In parental cells, GFP-Atg8p was fully degraded by trypsin, indicating it was readily accessible in the cytosol. This means GFP-Atg8p enclosed in autophagosomes is rapidly converted to free GFP in parental cells due to the rapid turnover of autophagosomes fusing

to the vacuoles. In *isc1*Δ cells, a small pool of GFP-Atg8p remained protease-protected, probably enclosed by a membrane as it was degraded in the presence of the detergent (Figure 3.S1). Together, these results suggest that disturbed autophagic flux of *isc1*Δ cells is not attributed to alterations in the fusion of vesicles to the vacuole but rather to impaired Pep4p-mediated proteolysis.

Defective processing of ApeI through the Cvt pathway may also result from alterations in vacuolar protein sorting and trafficking, which is a crucial part of autophagy regulation. To assess if *isc1*Δ cells exhibit vesicular trafficking defects, we have evaluated whether CPY secretion is altered in *isc1*Δ cells. Several vacuolar protein sorting (*vps*) mutants that fail to deliver CPY to the vacuole display an aberrant secretion of CPY (Rothman & Stevens, 1986, Raymond *et al.*, 1992). Our results show that *isc1*Δ cells, in contrast to parental cells, exhibited CPY secretion, similarly to the *vps4*Δ mutant (Figure 3.2.D). Taken together, these data indicate that the accumulation of prCPY in *isc1*Δ cells may arise not only from defective Pep4p-mediated processing but also from impaired Golgi-to-vacuole trafficking. The mutual observation that the Cvt pathway and CPY trafficking are affected in *isc1*Δ cells, already under basal conditions, indicates that vesicular trafficking may contribute to reduced autophagic flux in this mutant strain. The overexpression of *VAM3* (Darsow *et al.*, 1997) and *YKT6* (Kweon *et al.*, 2003), which are involved in endocytic trafficking and vacuolar docking/fusion, had no effect on Cvt pathway and CPY secretion (Figure 3.S2.A and 3.S2.B), suggesting that the trafficking defect in *isc1*Δ cells does not occur at a step involving Vam3p and Ykt6p (t-SNARE/v-SNARE pairing).

Proper vacuolar acidification has been shown to be pivotal for autophagy as it is required for the activation of vacuolar proteases (Nakamura *et al.*, 1997) and the autophagosome-vacuole fusion step (Abeliovich *et al.*, 2000). This led us to assess whether vacuolar acidification is affected in *isc1*Δ cells by using the fluorescent weak base quinacrine, whose accumulation preferentially relies on acidic compartments. The results show that quinacine staining in *isc1*Δ cells was lower to that of parental cells (Figure 3.2.E). In agreement with these results, by measuring concanamycin A-sensitive ATPase activity in isolated vacuoles, we also show that *isc1*Δ vacuoles exhibited lower V-ATPase activity than wild-type vacuoles (Figure 3.2.F), indicating that *isc1*Δ cells display defective vacuolar acidification.

3.2.2. The Sit4p protein phosphatase and the TORC1-Sch9p pathway contribute to vesicular trafficking and vacuolar defects of *isc1*Δ cells

The ceramide-activated protein phosphatase Sit4p was previously implicated in *isc1*Δ phenotypes (Barbosa *et al.*, 2011). Therefore, we analyzed whether Sit4p underlines the autophagic defects of this mutant. The deletion of *SIT4* in *isc1*Δ cells increased GFP-Atg8p processing (Figure 3.3.A) and the maturation of prApeI (Figure 3.3.B) after rapamycin treatment. The deletion of *TOR1* or *SCH9*, which suppresses the reduced autophagic flux of *isc1*Δ cells (Teixeira *et al.*, 2014), also increased prApeI maturation in this mutant (Figure 3.S3.A).

Next, we assessed if Sit4p and the TORC1-Sch9p pathway are also implicated in vesicular trafficking and vacuolar defects observed in *isc1*Δ cells. Lower Pep4p activity, the exacerbated CPY secretion and the defective vacuolar acidification exhibited by *isc1*Δ cells were abolished in *isc1*Δ*sit4*Δ double mutants (Figure 3.3.C-E), as well as in *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells (Figure 3.S3.B-D).

Isc1p-deficient cells displayed trafficking defects and vacuolar fragmentation associated with impaired autophagy, similar to *vam3*Δ (Darsow *et al.*, 1997) and *ypt7*Δ mutants (Kim *et al.*, 1999). Notably, the restoration of the autophagic flux was also associated with the suppression of vacuolar fragmentation in *isc1*Δ*sit4*Δ, *isc1*Δ*sch9*Δ cells and to a lesser extent, in *isc1*Δ*tor1*Δ cells (Figure 3.S4). Together, these results are consistent with the idea that the protein machinery involved in vesicular trafficking events plays an important role in the modulation of autophagy in the mutant strain.

We have previously shown that *isc1*Δ mutants have increased levels of some ceramide species coupled with the activation of Sch9p and Sit4p and we proposed that the TORC1-Sch9p signalling pathway acts as a central axis to integrate upstream Isc1p-driven ceramide signalling signals to downstream effectors Sch9p and Sit4p (Teixeira *et al.*, 2014). Combined with the results presented here, this suggests that ceramide plays a regulatory role in autophagy.

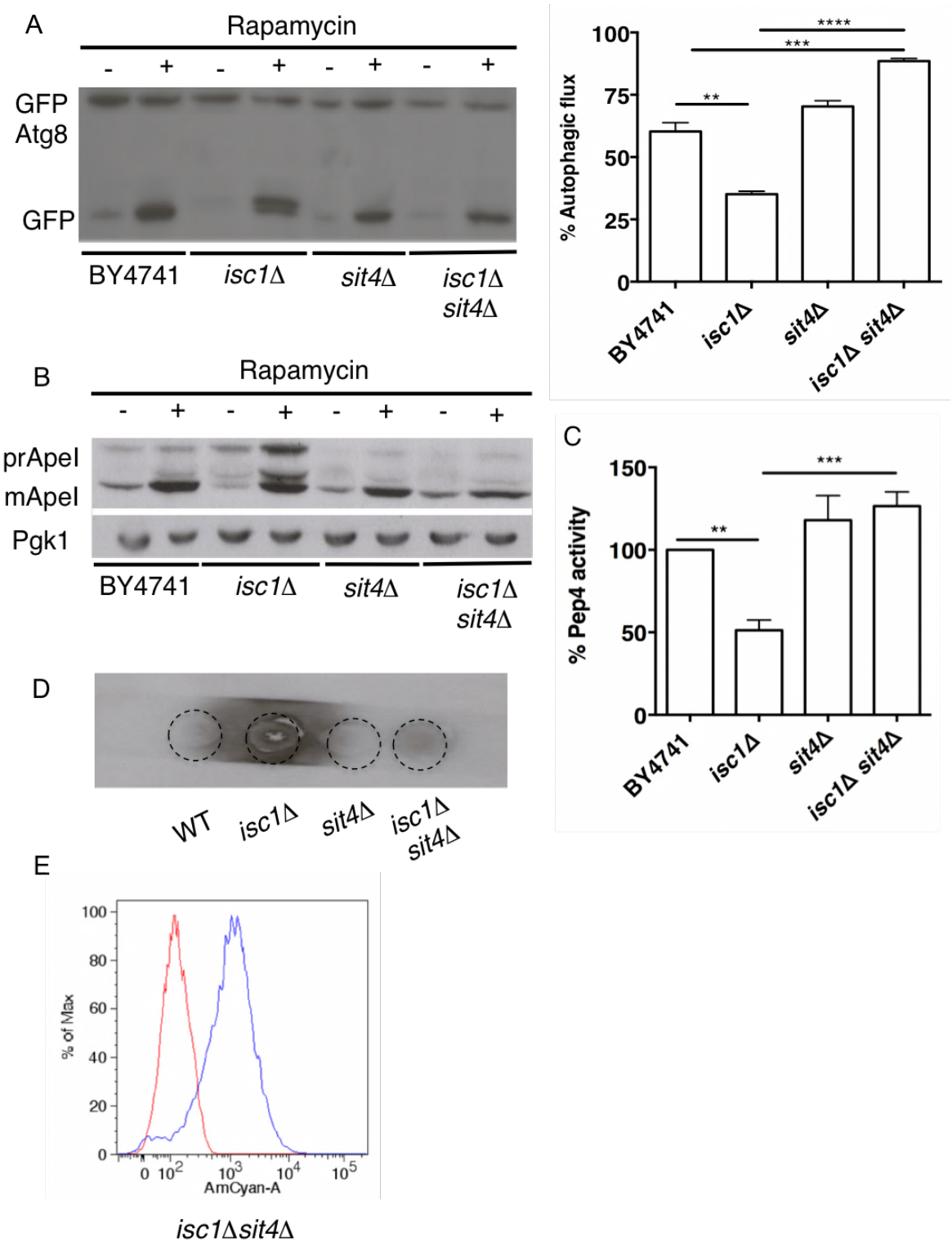

Figure 3.3. The deletion of *SIT4* suppressed Pep4p-dependent vacuolar proteolysis and the vacuolar protein sorting defects in *isc1Δ* cells.

A. BY4741, *isc1*Δ, *sit4*Δ and *isc1*Δ*sit4*Δ cells carrying GFP-Atg8p were grown in SC-medium to exponential phase and treated with either DMSO (vehicle) or rapamycin (200 ng/mL) for 3h. Proteins were analyzed by immunoblotting, using anti-GFP antibody. The quantification of autophagic flux (GFP/(GFP+GFP-Atg8p)) is shown. ****p<0.0001;

***p<0.001; **p<0.01. **B.** The processing of the Cvt pathway prApeI to mApeI was analyzed by immunoblotting in BY4741, *isc1*Δ, *sit4*Δ and *isc1*Δ*sit4*Δ cells treated as described in 3A. **C.** Specific Pep4p activity was measured in protein extracts of BY4741, *isc1*Δ, *sit4*Δ and *isc1*Δ*sit4*Δ cells grown to exponential phase in SC-medium. Values were normalized to parental cells (set as 100%). Values are mean ± SD of at least three independent experiments. ***p<0.001; **p<0.01. **D.** CPY secretion was analyzed in BY4741, *isc1*Δ, *sit4*Δ and *isc1*Δ*sit4*Δ cells as described in figure 3.2.D. **E.** Vacuolar acidification of *isc1*Δ*sit4*Δ cells was analyzed using quinacrine staining, as described in 3.2.E. Unprobed and probed cells are depicted in red and blue, respectively. Representative histograms are shown.

To explore this hypothesis, we enzymatically decreased ceramide load in *isc1*Δ cells by ectopically increasing *YPC1* ceramidase expression (Mao *et al.*, 2000). Overexpression of *YPC1* efficiently restored Pep4p activity in *isc1*Δ cells to that of WT values (Figure 3.S5.A), but did not suppress the CPY vacuolar protein sorting defect (Figure 3.S5.B). This indicates that decreasing ceramide substrates of Ypc1p can overcome some but not all the mechanisms deregulated in the *isc1*Δ mutant. Taken together, our data suggest that ceramide species increased in *isc1*Δ cells signal through Sit4p and the TORC1-Sch9p pathways and impair vesicular trafficking, vacuolar function (proteolysis and acidification) and morphology, ultimately contributing to reduced autophagic flux in the mutant strain.

Mechanisms other than autophagy are involved in cellular protein turnover. It is well known that autophagy is coordinated with proteasomal degradation and the unfolded protein response (UPR) signalling to govern cell fate, either by restoring protein homeostasis or inducing cell death when the folding capacity is overwhelmed (Korolchuk *et al.*, 2010, Salminen & Kaarniranta, 2010). Isc1p-deficient cells are sensitive to tunicamycin (Kim *et al.*, 2012), a hallmark of defects in ER stress response. We evaluated the activation of an UPRE::LacZ reporter in cells exposed to tunicamycin and DTT, two well-established ER stress inducers. The *isc1*Δ cells showed an UPRE-regulated induction comparable to the observed in parental cells (Figure 3.S6.A).

It was previously reported that the ceramide-activated protein phosphatase Sit4 negatively regulates the UPR in response to ceramide (Mousley *et al.*, 2008). Since very long chain ceramide species levels increased during aging in *isc1*Δ

cells, which were previoulsy implicated in the activation of Sit4p, we hypothesized that UPR could be innapropriately downregulated in *isc1*Δ cells by activating Sit4p. We observed that UPR signalling was decreased both in exponential phase and during aging by a Sit4p-dependent mechanism (Figure 3.S6.B). However, the overexpression of *HAC1* (encoding for the transcription factor that regulates the UPR) had minor effects on *isc1*Δ CLS and failed to abolish the hydrogen peroxide sensitivity of these mutants (Figure 3.S6.C-D). It is unlikely that lower UPR levels during aging of *isc1*Δ cells are associated with lower ER stress since the overexpression of *HAC1* partially extended the CLS of *isc1*Δ cells. Instead, the results suggest that failure to induce UPR during aging results from inapropriate activation of Sit4p in response to deranged ceramide levels (Barbosa *et al.*, 2011). In addition, the ER-associated degradation mechanism (ERAD) was not compromised in *isc1*Δ cells (Figure 3.S6.E). These results suggest that changes in the UPR do not significantly contribute to *isc1*Δ phenotypes.

3.2.3. Mitophagy is hyperactivated in Isc1p-deficient cells

Since *isc1*Δ cells present mitochondrial dysfunction and fragmentation (Teixeira *et al.*, 2014), we hypothesized that mitophagy is triggered to counteract mitochondria loss-of-function in the mutant. The induction of mitophagy was quantitatively measured by employing a modified alkaline phosphatase (mtPho8) assay (Sampaio-Marques *et al.*, 2012). First, we have monitored mitophagy in cells incubated in lactate medium (post-log phase), as described (Kanki *et al.*, 2009b). The growth defect of *isc1*Δ cells in this medium is less pronounced than that observed in acetate or glycerol medium and cells are still able to grow within this period of time (48 hours), as reported (Vaena de Avalos *et al.*, 2004) (and our observations). In parental cells, alkaline phosphatase activity increased 2-fold after shifting cells from glucose to lactate medium. This variation was higher (3-fold) in *isc1*Δ cells (Figure 3.4.A). This indicates that mitophagic activity (the uptake of mitochondria through mitophagy) is enhanced in *isc1*Δ cells. We also observed mitochondrial fragmentation and reduced oxygen consumption in *isc1*Δ cells under the same conditions (Figure 3.S7).

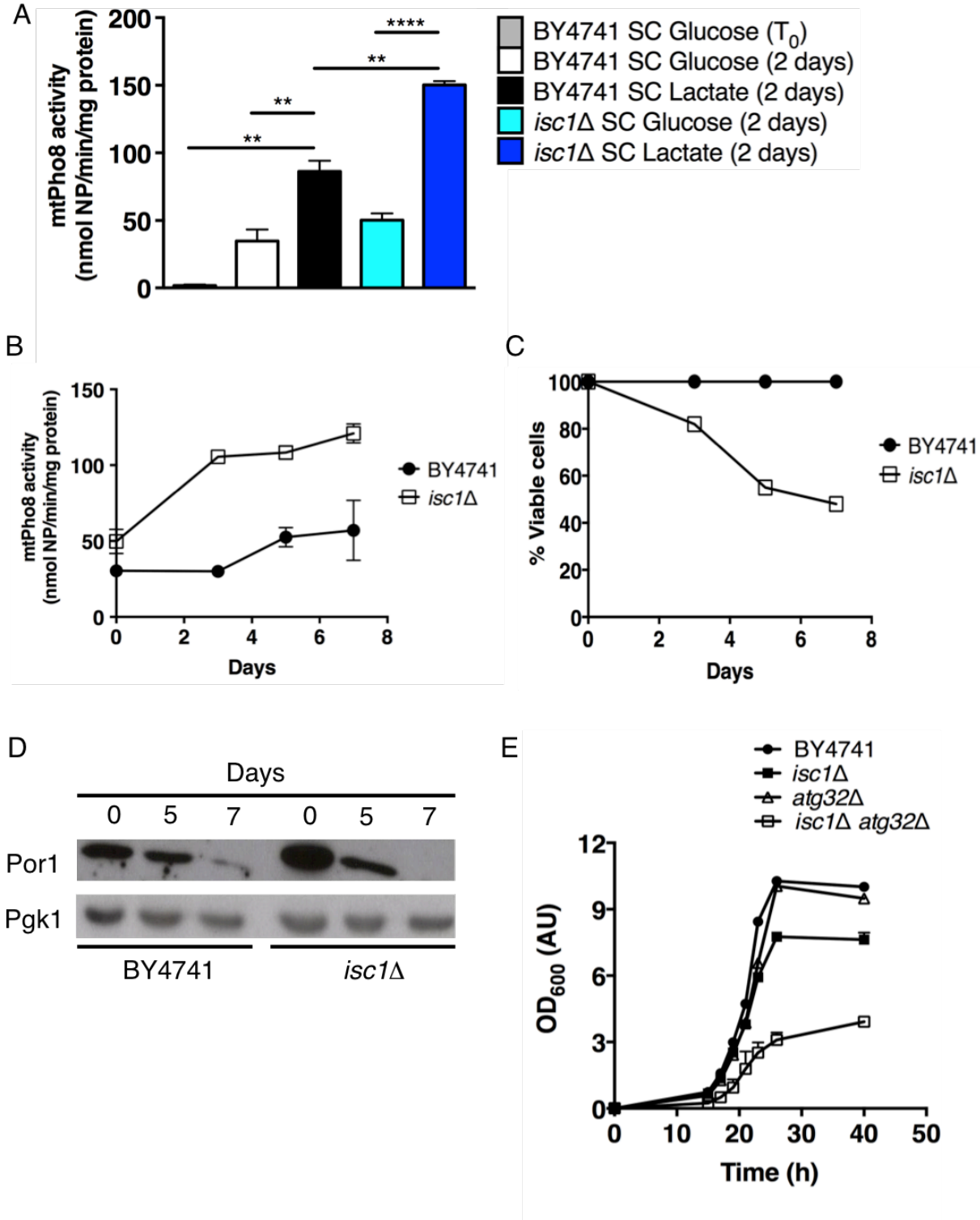

Figure 3.4. The hyperactivation of mitophagy was correlated with decreased cell viability in *isc1*Δ cells.

A. Mitophagic activity was measured by performing the alkaline phosphatase assay. BY4741 and *isc1*Δ cells expressing inactive Pho8 proenzyme targeted to the mitochondrial matrix (mtPho8) were grown in SC-glucose (OD_{600}=0.1, T_0) and either maintained in the same medium or shifted to SC-lactate medium for 48 h. Alkaline phosphatase activity was measured in glucose (before the shift) and in glucose or lactate-

growing cells after 2 days. Values are mean ± SD of at least three independent experiments. ****p<0.0001; **p<0.01. **B.** BY4741 and *isc1*Δ cells carrying mtPho8 were grown in SC-glucose to PDS phase, washed twice, and incubated in water during aging. Mitophagy activity was measured as described. Values are mean ± SD of at least three independent experiments**. C.** BY4741 and *isc1*Δ cells carrying mtPho8 were grown as described in 4C. Cell viability was expressed as the percentage of the colony-forming units (aged cells versus day 0 cells at PDS phase). Values are mean ± SD of at least three independent experiments. **D**. Mitochondrial Por1p protein levels were monitored by immunoblotting in BY4741 and *isc1*Δ cells grown as described in 4C, using anti-Por1p (top panel). Pgk1p was used as loading control. A representative blot of at least three independent experiments is shown. **E.** Growth curves of BY4741, *isc1*Δ, *atg32*Δ and *isc1*Δ*atg32*Δ cells grown in SC-glucose medium. Values are mean ± SD of three independent experiments.

We have monitored mitophagy induction during aging (in CR conditions by shifting to water). We observed a slight increase in alkaline phosphatase activity in parental cells at day 5; however, mitophagy was induced more than 2-fold in *isc1*Δ cells already at day 3 and remained at higher steady levels up to day 7 (Figure 3.4.B). This shows that higher induction of mitophagy is also observed at post-log phase in CR condtions (water) in *isc1*Δ cells. Importantly, this was correlated with loss of cell viability in *isc1*Δ cells (Figure 3.4.C).

We have also analyzed total levels of Por1p (Figure 3.4.D), a mitochondrial outer membrane protein, as a means to monitor mitochondrial turnover during aging. We observed increased Por1p levels already at PDS phase (T_0) in *isc1*Δ cells, which correlates with increased mitochondrial mass measured by flow cytometry (Figure 3.S8). At day 5, there was a slight decrease in Por1p content in parental cells, which related well with the induction of mitophagy (Figure 3.4.D). However, the decrease in Por1p levels was more pronounced in *isc1*Δ cells, which is consistent with higher induction of mitophagy within the same period of time (Figure 3.4.B).

To evaluate if the hyperactivation of mitophagy is advantageous for the mutant strain, we analyzed if abolishing this process by deleting *ATG32* affected cell growth of Isc1p-deficient cells. As shown in Figure 3.4.E, the growth defect of *isc1*Δ cells upon transition to the PDS phase was significantly exacerbated in *isc1*Δ*atg32*Δ cells. The deletion of *ATG32* also shortened the CLS of *isc1*Δ cells

(Figure 3.S9). These results indicate a negative genetic interaction between *ISC1* and *ATG32* (Figure 3.4.E), and suggest that mitophagy is required to transiently counteract mitochondria loss of function in *isc1*Δ cells.

3.2.4. Mitochondrial fragmentation is associated with increased Dnm1p-dependent fission in *isc1*Δ cells

We next sought to assess changes in mitochondrial dynamics, which may contribute to the mitochondrial fragmentation and dysfunction of *isc1*Δ cells. We first analyzed whether mitochondrial fusion was affected by performing a standard mitochondrial yeast mating assay (Jensen *et al.*, 2000). Mata cells expressing mtDsRed were mated with Matα cells expressing mtGFP and the formation of diploids was monitored by microscopy (Figure 3.5.A). In parental cells, we observed a complete sumperimposition of both mitochondrial networks in the diploid cell, rendering a yellow signal. In the mutant strain, we observed that the majority of cells presented an almost complete merge of mitochondrial networks but some cells exhibited small portions that remained green or red, suggesting that mitochondrial fusion is slightly impaired in *isc1*Δ cells. The overexpression of *FZO1*, which encodes for a mitochondrial fusion protein, in *isc1*Δ cells partially restored a tubular shape (*circa* 50% of the analyzed cell population), resembling the normal mitochondrial network of parental cells (Figure 3.S10). We have also monitored the fission machinery by assessing Fis1p and Dnm1p levels in parental and *isc1*Δ cells grown to PDS phase (Figure 3.5.B). Although Fis1p levels were similar in both strains, Dnm1p levels were increased in *isc1*Δ cells compared to WT cells. These observations suggest that mitochondrial fragmentation in *isc1*Δ cells is due to mild defective fusion concomitant with enhanced Dnm1p-mediated fission events.

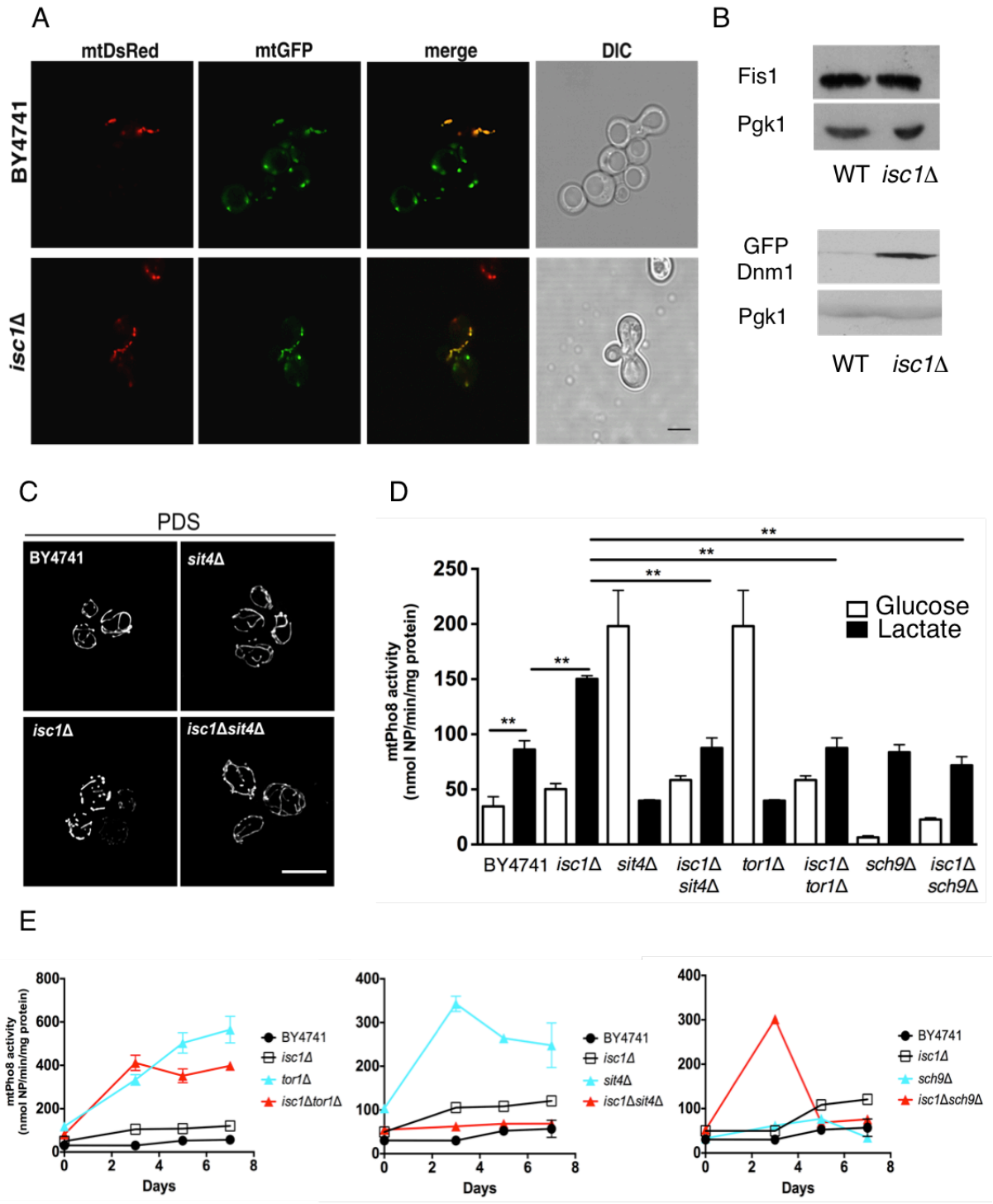

Figure 3.5. Mitochondrial fragmentation occurs by Dnm1p-dependent mechanisms and the deletion of *TOR1*, *SCH9* and *SIT4* suppressed mitochondrial fragmentation and this was correlated with proper restoration of mitophagy in *isc1*Δ cells under respiratory conditions.

A. Haploid BY4741 and *isc1*Δ cells with different mating types carrying mitochondrial-targeted GFP (pVT100U) and mtDsRed proteins were grown to the exponential phase and mated. The fusion of the mitochondrial networks was analyzed by fluorescence microscopy. Representative images are shown. Scale bar: 5 μm. **B.** Fis1p and Dnm1p-

GFP protein levels were monitored by immunoblotting in BY4741 and *isc1*Δ cells grown to PDS phase, using anti-Fis1p and anti-GFP, respectively (top panel). Pgk1p was used as loading control. A representative blot of at least three independent experiments is shown. **C.** BY4741, *isc1*Δ*, sit4*Δ and *isc1*Δ*sit4*Δ cells carrying mtDsRed were grown to PDS phase and mitochondrial network was analyzed by fluorescence microscopy. Representative images are shown. Scale bar: 5 μm. **D.** BY4741, *isc1*Δ*, sit4*Δ*, isc1*Δ*sit4*Δ, *tor1*Δ, *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells carrying mtPho8 were grown as described in figure 3.4.A. Mitophagy activity was measured by the alkaline phosphatase assay in glucose (open bars) or lactate-growing cells (closed bars) after 48 hours. Values are mean ± SD of at least three independent experiments. **p<0.01. **E.** BY4741, *isc1*Δ*, sit4*Δ*, isc1*Δ*sit4*Δ, *tor1*Δ, *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells carrying mtPho8 were grown in SC-glucose to PDS phase, washed twice, and incubated in water during aging. Mitophagy activity was measured as described in D. Values are mean ± SD of at least three independent experiments.

3.2.5. Deletion of *SIT4*, *TOR1* and *SCH9* suppresses mitochondrial fragmentation and deregulated mitophagy in *isc1*Δ cells

Apart from *TOR1* and *SCH9* disruption (Teixeira *et al.*, 2014), the deletion of *SIT4* also abolished mitochondrial fragmentation in *isc1*Δ cells (Figure 3.5.C). Sch9p acts upstream of Hog1p in response to ceramide signalling and the deletion of *HOG1* restores proper mitochondrial function to the mutant strain (Barbosa *et al.*, 2012). Moreover, Hog1p has been implicated in the regulation of mitophagy (Mao *et al.*, 2011). Thus, we also analyzed whether the Hog1p kinase contributes for these phenotypes. We observed that the normal tubular mitochondrial network of parental cells was restored in *isc1*Δ*hog1*Δ cells (Figure 3.S11.A). Next, we monitored mitophagy in lactate-grown cells. Mitophagy was induced to lower extent in *isc1*Δ*sit4*Δ, *isc1*Δ*tor1*Δ, *isc1*Δ*sch9*Δ cells (Figure 3.5.D) and *isc1*Δ*hog1*Δ mutants (Figure 3.S11.B) compared to *isc1*Δ cells, suggesting that mitochondrial dynamics and mitophagy are coupled in *isc1*Δ cells upon respiratory conditions. We have also analyzed mitophagy during aging of these mutants in water (Figure 3.5.E and 3.S11.C). Though the individual kinetics varied, it was apparent that the induction of mitophagy was lower at late stages of the aging process (after 5 days) in *isc1*Δ*sch9*Δ and *isc1*Δ*sit4*Δ cells but not in *isc1*Δ*tor1*Δ and *isc1*Δ*hog1*Δ cells (Figure 3.5.E and 3.S11.C) relative to *isc1*Δ single mutants.

3.2.6. The deletion of *DNM1* abolishes mitochondrial fragmentation, impaired mitophagy, mitochondrial dysfunction, hydrogen peroxide sensitivity and shortened CLS of *isc1*Δ cells

To provide more evidence that mitochondrial fragmentation and the hyperactivation of mitophagy are coupled in response to mitochondrial loss-of-function in *isc1*Δ cells, we assessed how the deletion of *DNM1* affected *isc1*Δ phenotypes. The deletion of *DNM1* suppressed mitochondrial fragmentation in Isc1p-deficient cells (Figure 3.6.A) to a hyperfused-like mitochondrial network similar to that of *dnm1*Δ cells. The induction of mitophagy was lower in lactate-grown *isc1*Δ*dnm1*Δ cells relative to *isc1*Δ cells (Figure 3.6.B), suggesting that Dnm1p-associated mitochondrial fragmentation leads to the activation of mitophagy in the mutant under respiratory conditions. To uncover if *DNM1* deletion suppressed other mitochondrial-related *isc1*Δ phenotypes, oxidative stress resistance and CLS were characterized. Notably, *dnm1*Δ mutants were more resistant to hydrogen peroxide and *DNM1* disruption suppressed the sensitivity of *isc1*Δ cells to this oxidant (Figure 3.6.C). The deletion of *DNM1* also promoted CLS extension in both parental and *isc1*Δ cells (Figure 3.6.D). The suppression of *isc1*Δ phenotypes by *DNM1* disruption was related to improved mitochondrial fitness. Indeed, the growth defect of *isc1*Δ cells on glycerol medium was also suppressed (Figure 3.6.E), both oxygen consumption and COX activity were increased in *isc1*Δ*dnm1*Δ cells by 6- and 17-fold, respectively (Figure 3.6.F-G) compared to *isc1*Δ cells, and the hyperpolarization of the *isc1*Δ mitochondria was abolished (Figure 3.6.H).

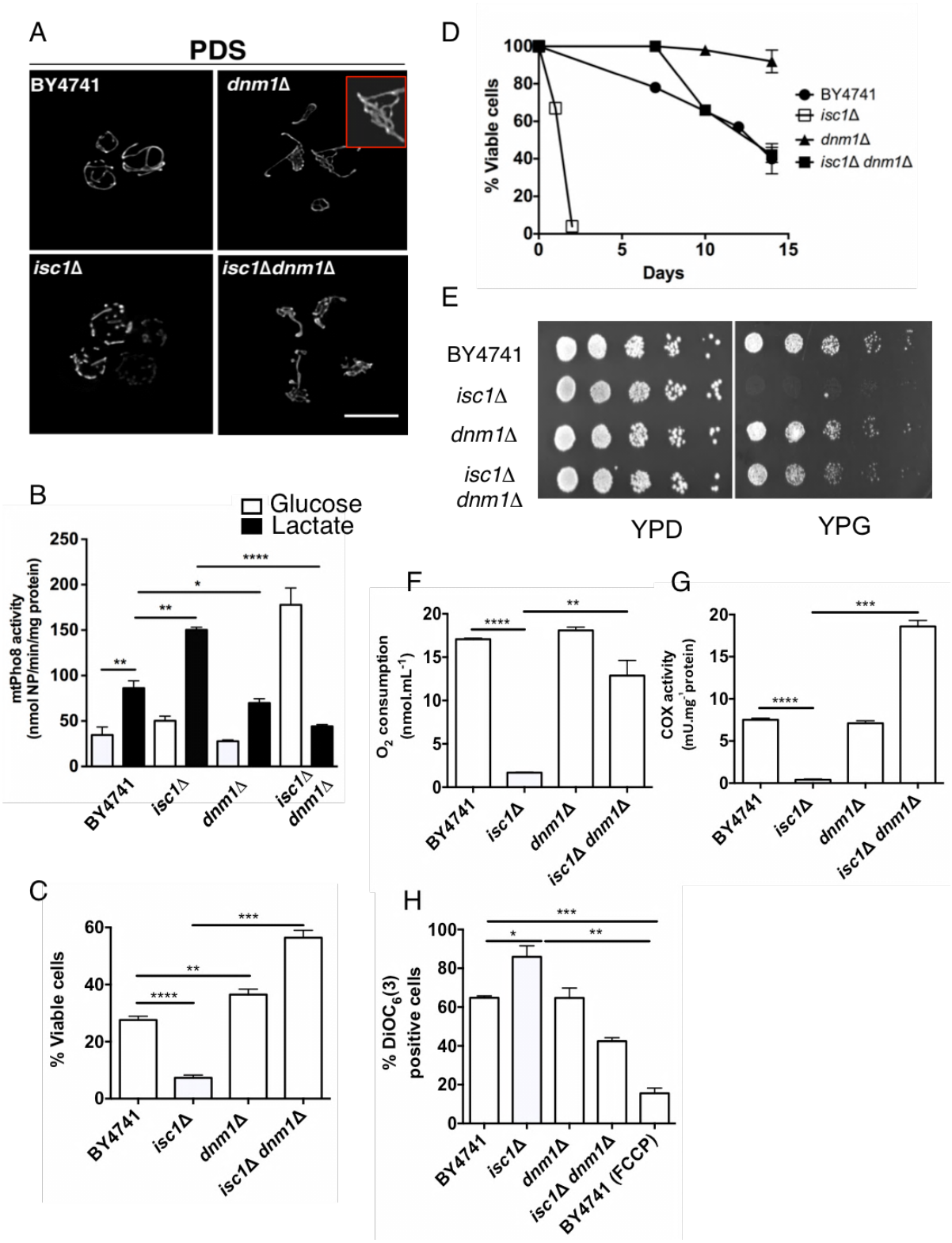

Figure 3.6. The deletion of *DNM1* abolishes the shortened CLS, oxidative stress sensitivity and mitochondrial dysfunctions displayed by *isc1*Δ cells.

A. BY4741, *isc1*Δ*, dnm1*Δ *and isc1*Δ*dnm1*Δ cells carrying mtDsRed were grown to PDS phase and mitochondrial network was analyzed by fluorescence microscopy. A detailed image of hyperfused mitochondria in *dnm1*Δ mutant is shown. Representative images are shown. Scale bar: 5 μm. **B.** Cells carrying mtPho8 were grown as described in figure

3.4.A. Mitophagy activity was measured by the alkaline phosphatase assay. Values are mean ± SD of at least three independent experiments. ****p<0.0001; **p<0.01; *p<0.05. **C.** Cells were grown in SC-medium to the exponential phase and exposed to 1.5 mM H_2O_2 for 60 min. Cell viability was expressed as the percentage of the colony-forming units (treated cells versus untreated cells). Values are mean ± SD of at least three independent experiments. ****p<0.0001; ***p<0.001; **p<0.01. **D.** Cells were grown in SC-glucose and kept in the medium at 26°C. The viability was determined as the percentage of the colony-forming units at time *T* in relation to T_0. Values are mean ± SD of at least three independent experiments. **E.** Cells were grown in SC-glucose to the exponential phase and then diluted to OD_{600}=0.1. Fivefold dilution series were spotted on YPD (glucose) or YPG (glycerol) medium and grown at 26°C for 4 days. **F.** Oxygen consumption rate was measured in cells grown to PDS phase. Values are mean ± SD of at least three independent experiments. ****p<0.0001; **p<0.01. **G.** Cytochrome *c* oxidase (COX) activity was determined in cells grown to the PDS phase. Values are mean ± SD of at least three independent experiments. ****p<0.0001; ***p<0.001. **H.** Cells were grown in SC-glucose to the PDS phase, stained with the potential-sensitive dye 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] for 30 min and analyzed by flow cytometry. Treatment of the parental strain (BY4741) with FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, 10 mM) was used as a positive control (depolarizing event). Values are mean ± SD of at least three independent experiments. ***p<0.001; **p<0.01; *p<0.05.

3.2.7. Isc1p interacts with Dnm1p

Our data showing that Isc1p regulates mitochondrial dynamics and function in a Dnm1p-dependent manner led us to investigate whether these proteins interact. For this purpose, we overexpressed FLAG-tagged Isc1p (under *GAL1* promoter) in yeast cells expressing endogenously C-terminally GFP-tagged Dnm1p and cells were grown under respiratory conditions (galactose, for 16-18h hours), where Isc1p is translocated from the ER to the mitochondria (Vaena de Avalos *et al.*, 2004). In fact, FLAG-Isc1p is localized at the ER after incubation for 4-6 hours on galactose, whereas longer incubation (24 h) leads to the mitochondrial localization of Isc1p (Vaena de Avalos *et al.*, 2004). Immunoprecipitation with a FLAG antibody co-precipitated Dnm1p-GFP in cells

expressing FLAG-Isc1p but not in cells expressing untagged Isc1p (Figure 3.7), indicating that Isc1p interacts with Dnm1p *in vitro*.

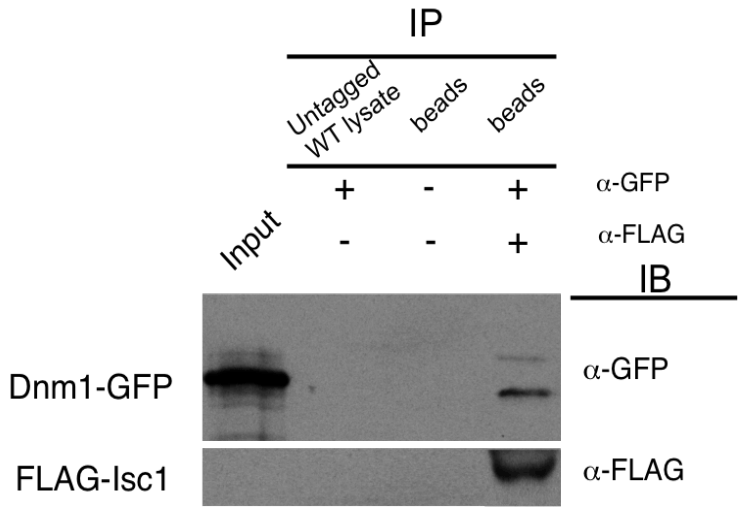

Figure 3.7. Isc1p interacts with Dnm1p *in vitro*. Lysates from cells expressing Dnm1p-GFP and FLAG-tagged Isc1p proteins were used for immunoprecipitation with either anti-GFP or anti-FLAG protein G beads. Cells were grown in SC-Galactose to induce FLAG-Isc1p and to monitor the interaction between these proteins under respiratory conditions, where Isc1p is known to translocate from the ER to mitochondria. Immunoprecipitated fractions were analyzed by SDS-PAGE and Western blotting. Lysate from untagged BY4741 (lane 2) and G protein beads (lane 3) were used as controls to evaluate unspecific binding of antibodies and beads to lysate proteins.

3.3. Discussion

The role of sphingolipids in aging and age-associated pathologies, such as neurodegenerative diseases and cancer, has been extensively reported. In fact, it is well established that alterations on sphingolipid rheostat have significant impact on cell growth and survival and general stress response with impact on the onset of these diseases. However, the molecular mechanisms by which signalling pathways are activated in response to alterations in sphingolipid biosynthesis or turnover are not yet fully understood. This clarification is pivotal to understand sphingolipid functions in more detail and define new strategies to improve human health and extend lifespan.

In this work, we report that Isc1p-driven ceramide signalling regulates macroautophagy and the Cvt pathway, mitophagy and mitochondrial dynamics. We show that *isc1*Δ cells present defects in autophagy flux, exacerbated mitophagy and increased mitochondrial fission, leading to mitochondrial network fragmentation. The impairment in autophagy was not associated with changes in vesicle nucleation. Instead, it resulted from primary defects observed in the mutant strain at later stages of autophagy, namely defective vesicular trafficking and Pep4p-mediated vacuolar proteolysis. The impairment of vesicular trafficking results from a general vacuolar protein sorting defect, since *isc1*Δ cells displayed aberrant secretion of CPY and disturbed processing of ApeI through the Cvt pathway. The lower Pep4p activity could be due to the lower vacuolar acidification and V-ATPase activity of *isc1*Δ cells, since Pep4p is not mislocalized in this mutant (our unpublished data). Notably, *PEP4* overexpression ameliorated impaired autophagic flux in *isc1*Δ cells, indicating that Pep4p-mediated vacuolar proteolysis is a limiting factor in the mutant. Consistent with a role in autophagy, Isc1p has been reported to interact with Aut5p, a protein involved in the lysis of intravacuolar vesicles (Epple *et al.*, 2003).

We have observed that the induction of GFP-Atg8p (in rapamycin treated cells) and basal Atg13p phosphorylation are not significantly altered in *isc1*Δ cells (Figure 3.3.A and 3.S12). Nevertheless, we cannot discard the possibility that autophagic induction may be altered in *isc1*Δ cells due to the hyperactivation of the TORC1 signalling pathway. It is possible that hyperactive TORC1 may target the Atg1p kinase activity, which is required for efficient association of Atg1p with Atg17p and Atg13p (Atg1p kinase complex) during autophagy. Moreover, Sch9p activation, which is increased in *isc1*Δ cells (Teixeira *et al.*, 2014), was shown to contribute to the inhibition of autophagy independently of phosphorylation of Atg13p (Yorimitsu *et al.*, 2007), which possibly accounts for reduced autophagic activity in *isc1*Δ cells. Yamagata *et al.* have recently reported that MIPC or $M(IP)_2C$ complex sphingolipids are not required for autophagy but reduced IPC causes autophagic defects, as the inhibtion of IPC synthesis by aureobasidin A (AbA) causes a reduction in autophagic activity (Yamagata *et al.*, 2011). Since Isc1p-deficient cells accumulate these sphingolipids, we may discard the possibility that complex sphingolipids may play a key role for the defects in autophagy observed in *isc1*Δ cells.

Our results show that the TORC1-Sch9p and Sit4p-mediated signalling pathways impair autophagic flux in *isc1*Δ cells since the deletion of *TOR1*, *SCH9* and *SIT4* suppressed the defects on vesicular trafficking, vacuolar proteolytic capacity, vacuolar acidification and the vacuolar fragmentation phenotype, ultimately contributing to restore proper autophagic flux and the integrity of the Cvt pathway in *isc1*Δ cells. We therefore provide novel evidence that ceramide may constitute a quality control reporter in the process via the ceramide-activated Sit4p protein phosphatase and the activation of the TORC1-Sch9p pathway in response to Isc1p-driven ceramide signalling. Consistently, alterations in ceramide turnover promoted by ectopic overexpression of ceramidase *YPC1* in *isc1*Δ cells improved vacuolar proteolysis. Recently, Sit4p was shown to interact with autophagy effectors, namely Atg18p (Ho *et al.*, 2002).

In this work, we also implicate ceramide as a signal that contributes to UPR silencing observed in *isc1*Δ cells, due to activation of Sit4p. This is supported by the restoration of the UPR in the double mutant *isc1*Δ*sit4*Δ strain during aging. Together, these results demonstrate that Sit4p links ceramide homeostasis to ER stress response and autophagy in *isc1*Δ cells by acting as a ceramide sensor whose result is the innapropriate downregulation of these important biological processes, in part due to derranged ceramide metabolism, which then alter cell signaling through Sit4p and the TORC1-Sch9p pathway. More recently, Sit4p has been shown to be present in different protein complexes. Indeed, Sit4p activity is regulated by TORC1 in a Tip41p/Tap42p manner by phosphorylation (Jacinto *et al.*, 2001) whereas it can be activated by ceramide in a Isc1p-dependent manner (Barbosa *et al.*, 2011). Luke *et al.* (Luke *et al.*, 1996) and Woodacre *et al.* (Woodacre *et al.*, 2013) have demonstrated that Sit4p also interacts with SAP proteins (SIT4-associated proteins), indicating some degree of response specificity. We propose that ceramide signalling acts as a quality control reporter ideally suited to sense the functional status of autophagy and the ER stress response via Sit4p-mediated mechanisms. Sit4p likely integrates signalling derived from both TORC1 and Isc1p-mediated ceramide homeostasis to regulate these processes and the response specificity may be acquired by interacting with different protein subunits, depending on the stimulus.

Importantly, Swinnen *et al*. have recently reported that ceramide levels are regulated by the TORC1-Sch9p signalling pathway at a transcriptional level

(Swinnen *et al.*, 2014). In particular, *sch9*Δ cells exhibit increased expression of *YPC1*. Therefore, the suppression of *isc1*Δ phenotypes imparted by *SCH9* deletion can be associated with decreased ceramide levels due to upregulation of *YPC1*, similarly to the supression of some autophagic defects by ectopic expression of *YPC1* reported in this study. We thus propose that Sit4p acts as a ceramide "sensor" and Sch9p may control the phosphatase activity by adjusting ceramide levels (through Ypc1p).

The molecular mechanisms underlying the regulation of mitophagy in response to alterations in sphingolipid metabolism remain largely unknown. We provide evidence that mitophagic activity is increased and associated with mitochondrial fragmentation and impaired mitochondrial function in *isc1*Δ cells when respiration is induced (lactate-growing conditions). This is consistent with an adaptive response to counteract mitochondrial dysfunction. The induction of mitophagy is increased during aging of *isc1*Δ cells, a phenotype that was associated with shortened CLS of the mutant strain. The deletion of *ATG32* aggravated the growth defect of *isc1*Δ cells, particularly upon the transition to PDS phase (respiratory metabolism), and further shortened its CLS, indicating that Atg32p is required for *isc1*Δ cell growth and that mitophagy contributes to survival of *isc1*Δ cells during stationary phase. Sentelle *et al.* have recently reported an interaction between ceramide and LC3B-II upon DNM1L/DRP1-dependent mitochondrial fission, which ultimately contributes to impaired mitochondrial function by inducing mitophagic cell death (Sentelle *et al.*, 2012). Isc1p-deficient cells also exhibit mitochondrial fragmentation associated with Dnm1p-dependent mechanisms and with the activation of Sit4p and the TORC1-Sch9p pathways in response to derranged ceramide load, leading to mitochondrial dysfunction.

Since mitophagy is hyperactivated in *isc1*Δ cells, could mitophagy be acting as an adaptive clearance mechanism in response to the segregation of dysfunctional mitochondria promoted by mitochondrial fragmentation? We observed that lactate-growing *isc1*Δ cells present mitochondrial fragmentation and mitochondrial dysfunction associated with increased induction of mitophagy. Importantly, the deletion of *TOR1*, *SCH9*, *SIT4* and *HOG1* lowers the induction of mitophagy when respiration is induced in *isc1*Δ cells (lactate) and this is associated with the restoration of mitochondrial network integrity and improvement of the organelle function (Barbosa *et al.*, 2011, Barbosa *et al.*, 2012, Teixeira *et al.*,

2014). Altogether, the results point out to a molecular mechanism in which the impairment of mitochondrial dynamics leads to the segregation of dysfunctional and damaged mitochondria, in a Dnm1p-dependent manner, to be degraded by mitophagy in *isc1*Δ cells upon respiratory metabolism (post-log phase, lactate). This hypothesis is supported by the fact that the deletion of *DNM1* reverted the mitochondrial fragmentation of *isc1*Δ cells, which was associated with lower activation of mitophagy in lactate-growing cells and overall improvement of mitochondrial function. Therefore, we propose that Dnm1p-mediated alterations in mitochondrial dynamics promote mitophagy, thus tipping the balance in favour of fission and thereby enforcing segregation of damaged/dysfunctional mitochondria to be removed by mitophagy in *isc1*Δ cells under respiratory conditions. This supports a functional link between mitophagy and mitochondrial dynamics and gives new insights on the role of ceramide in the crosstalk between these important mitochondrial quality control mechanisms. In fact, our data supports that Isc1p-driven ceramide signaling also plays a regulatory role in the regulation of mitophagy and mitochondrial dynamics in yeast by modulating the activation of the TORC1-Sch9p pathway, Sit4p and the HOG pathway, which responds to ceramide in a Sch9p-dependent manner, under respiratory conditions (post-log phase). We propose that, when Isc1p is post-translationally activated during the transition to a respiratory metabolism and relocalizes to mitochondria, the protein may adjust ceramide pools to regulate the activation of these signalling pathways and therefore contribute to proper mitochondrial function and network organization in yeast.

During aging, the induction of mitophagy further increased in *isc1*Δ*tor1*Δ and *isc1*Δ*hog1*Δ cells, comparing with *isc1*Δ cells. In contrast, it was decreased in *isc1*Δ*sit4*Δ and *isc1*Δ*sch9*Δ cells at late stages of the aging process (in water), rendering the strongest phenotypes, which is consistent with the fact that Sch9p and Sit4p integrate ceramide signalling with TORC1 readouts. Nevertheless, these results do not allow to clearly assess the role of mitophagy in aging of *isc1*Δ cells.

We also demonstrate, for the first time, that a protein involved in sphingolipid metabolism, namely Isc1p, interacts with the mitochondrial fission machinery protein Dnm1p under respiratory growth conditions. We speculate that Isc1p-driven ceramide signalling may regulate Dnm1p expression and activity. This is supported by the fact that *ISC1* disruption leads to the fragmentation of the

network by Dnm1p-dependent mechanisms, as demonstrated in this study. Kitagaki *et al*. have reported that a decrease in the levels of α-hydroxylated phytoceramide was associated with mitochondrial dysfunction in *isc1*Δ cells (Kitagaki *et al.*, 2007). Therefore, we hypothesize that alterations in ceramide content (dependent of Isc1p activity) leading to dysfunction of mitochondria may constitute a signal that promotes the recruitment of Dnm1p, leading to the segregation and degradation of damaged mitochondria by mitophagy in yeast. In agreement with this hypothesis, Parra *et al.* have reported that C_2-ceramide induced mitochondrial fragmentation associated with increased mitochondrial DRP1 and FIS1 content, DRP1 colocalization with FIS1, and early activation of apoptosis (Parra *et al.*, 2008).

In mammalian cells, ceramide triggers autophagy by inactivating the mTOR-signalling pathway downstream of AKT/PKB (Ravikumar *et al.*, 2004). Furthermore, ceramide also acts by activating the c-Jun N-terminal kinase 1 (JKAMP/JNK1), an orthologue of yeast Hog1p (Galcheva-Gargova *et al.*, 1994), which phosphorylates BCL-2 leading to its dissociation from BECN1/BECLIN1 (Pattingre *et al.*, 2009). C_2-Cer has also been shown to relieve IL-13-mediated inhibition of autophagy, whose mechanism appears to be mediated by the class I PI3K/AKT pathway (Petiot *et al.*, 2000). Additionally, recent work has shown that amino acid deprivation caused an increase in acid sphingomyelinase activity leading to enhanced ceramide content, which in turn promoted the PP1/PP2A-dependent inactivation of mTOR to induce autophagy (Taniguchi *et al.*, 2012). Our data supports that Isc1p-driven ceramide signalling is also important for proper induction of autophagy; however, autophagy is inhibited upon increased ceramide content observed in *isc1*Δ cells. Importantly, ceramide acts by downregulating the mTOR signalling in mammals, whereas the TORC1 pathway is hyperactive in *isc1*Δ cells, contributing to impaired autophagy induction and flux, in parallel to ceramide-mediated activation of Sit4p and Sch9p. One explanation for the apparently divergent regulation of autophagy between mammalian and yeast cells may rely on the fact that specific ceramide species are altered in Isc1p-deficient cells, namely very long chain ceramide species (Barbosa *et al.*, 2011), which in turn inappropriately activates signalling pathways responsive to these particular ceramide pools. Yeast and mammals, however, appear to retain similar molecular

mechanisms regarding the regulation of mitophagy and mitochondrial dynamics in response to ceramide signalling, as suggested by our study.

Overall, our results implicate Isc1p and ceramide signalling in the regulation of autophagy and support a model in which ceramide load promotes Dnm1p-driven mitochondrial fragmentation, therefore contributing to mitochondrial dysfunction and cell death in yeast. Importantly, our study provides mechanistic details and novel evidence that the modulation of ceramide metabolism by targeting these ceramide-responsive signalling pathways, which are conserved in mammals, could represent a novel strategy to improve lifespan and human health.

3.4. Experimental Procedures

3.4.1. Yeast strains and growth conditions

S. cerevisiae BY4741 was the parental strain of all haploid derivatives used in this study (Table 3.5.1). Yeast cells were grown aerobically at 26°C in a gyratory shaker (at 140 r.p.m.), with a ratio of flask volume/medium volume of 5:1. The growth media used were YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) glucose], YPL [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) lactate, pH 5.5], YPGlycerol [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 4% (v/v) glycerol], synthetic complete (SC) drop-out medium containing 2% (w/v) glucose, 0.67% yeast nitrogen base without amino acids (Difco Laboratories) and supplemented with appropriate amino acids (80 mg histidine L^{-1} (Sigma-Aldrich), 400 mg leucine L^{-1} (Sigma-Aldrich), 80 mg tryptophan L^{-1} (Sigma-Aldrich) and 80 mg uracil L^{-1}, (Sigma-Aldrich), SC drop-out medium containing 3% (v/v) glycerol and SC drop-out medium containing 2% (v/v) lactate, pH 5.5. Gene disruption was done by conventional methods and the proper integration of the cassettes was confirmed by PCR. The double mutant *isc1*Δ*atg32*Δ was generated by sporulation of the diploids resultant from mating *atg32*Δ::*KanMX4* carrying p*ISC1* and *isc1*Δ::*LEU2* strains, followed by tetrad dissection on SC-URA medium to select for spores carrying p*ISC1*, by standard techniques. The identities of the resulting strains were confirmed by growth on respective selective media.

3.4.2. Stress resistance and chronological lifespan

For H_2O_2 resistance assay, cells were grown to the exponential phase (OD_{600} = 0.6) and exposed to 1.5 mM H_2O_2 (Merck) for 60 min. The CLS assay was performed as described (Teixeira *et al.*, 2014).

3.4.3. Enzymatic activities and oxygen consumption

For the alkaline phosphatase assay, cells were harvested and ressuspended in 200 µL assay buffer [250 mM Tris (AMRESCO), 10 mM $MgSO_4$ (Sigma-Aldrich), 10 mM $ZnSO_4$ (Prolabo), pH 9.0 containing protease inhibitors (Complete Mini EDTA-free protease cocktail inhibitor tablets, Boehringer Mannhein). The cells were lysed by vortexing with glass beads and cell debris was removed by centrifugation at 13,000 r.p.m. for 15 min. 10 µg of extract was added to a final volume of 500 µL (assay buffer containing 1.13 mM nitrophenylphosphate (Sigma Fast, Sigma-Aldrich), and samples were incubated for 15 min at 30°C before terminating the reaction by adding 500 µL of stop buffer [2 M glycine (Nzytech)/NaOH (Merck), pH 11.0]. Production of nitrophenol was monitored spectrophotometrically (UV Mini 1240, Shimadzu), and its concentration was calculated using ε400 = 18,000 $M^{-1}.cm^{-1}$. The activity of COX (Poyton *et al.*, 1995), β-galactosidase (Teixeira *et al.*, 2014) and Pep4p (Jones *et al.*, 1982) was determined as previously described. For ER stress assays, tunicamycin (Sigma-Aldrich) and DTT (Nzytech) were used at indicated concentrations. For V-ATPase activity, ATP-dependent proton uptake activity was assayed by spectrophotometry using acridine orange (Sigma-Aldrich) (491-540 nm). Protein (15 µg) of isolated vacuoles were added to the reaction mixture containing 20 mM MOPS (Sigma-Aldrich)-Tris pH 7.0, 15 mM KCl (VWR), 135 mM NaCl (Merck) and 15 mM acridine orange followed by addition of 0.1 mM MgATP (Sigma-Aldrich). The reaction was stopped by the addition of 10 µL of 1 mM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma-Aldrich). Concanamycin A (0.1 µM, Enzo) was used a control to assess that V-ATPase was the main source of the vacuolar proton motive force. Protein content was determined by the method of Lowry, using bovine serum albumin (Nzytech) as a standard. Oxygen consumption rate was measured for 3 x 10^8 cells in PBS buffer (pH 7.4), using an

oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software.

3.4.4. Western Blot analysis

Cells were harvested and then ressuspended in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors and phosphatase inhibitors [50 mM sodium fluoride (Merck), 5 mM sodium pyrophosphate (Fluka Biochemika), 1 mM sodium orthovanadate (Sigma, Aldrich)]. Total protein extracts were lysed and centrifuged at 13,000 r.p.m. for 15 min and protein content was determined by the method of Lowry. Proteins were then analyzed by SDS-PAGE using 10% polyacrylamide (Sigma-Aldrich) gels and blotted onto a nitrocellulose membrane (GE Healthcare) and the Lumigen HRP chemiluminescent substrate (GE Healthcare). Assay for ERAD was done as reported (Ellis *et al.*, 2004). To evaluate Dnm1p-GFP protein levels and Atg13p phosphorylation, cells were harvested and subjected to alkaline lysis. Proteins (50 µg) were resolved by SDS-PAGE using 8.5% polyacrylamide gels. The primary antibodies used were rabbit anti-Atg13p and rabbit anti-ApeI (kindly provided by Dr. Klionsky), mouse anti-GFP (Roche, Basel, 11 814 460 001), mouse anti-CPY (Molecular Probes), mouse anti-Pgk1p (Invitrogen), rabbit anti-HA (Sigma-Aldrich), rabbit anti-actin (Sigma-Aldrich), mouse anti-Por1p (Molecular Probes) and rabbit anti-Fis1p (Santa Cruz Biotechnology, Inc.). The secondary antibodies used were anti-mouse IgG-peroxidase (Molecular Probes) and goat anti-rabbit IgG-peroxidase (Sigma-Aldrich).

3.4.5. Protease protection assay

Rapamycin-treated cells (40 OD_{600} units) were harvested at 4,000 r.p.m. for 5 min, washed twice with water, and incubated for 20 min at 30ºC in 4 mL of buffer A [100 mM Tris/H_2SO_4 (Sigma-Aldrich), pH 9.4] containing 20 mM dithiothreitol (DTT, Sigma-Aldrich). The cells were then pelleted, ressuspended in 4 mL of zymolyase buffer [1 M sorbitol (Sigma-Aldrich), 20 mM PIPES (AMRESCO), pH 6.8 containing 1.2 mg zymolyase 20T (Amsbio)] and spheroplasted for 30 min at 30°C. The spheroplasts were harvested at 2,000 r.p.m. for 5 min and hypotonically

lysed in PS200 buffer (200 mM sorbitol, 5 mM $MgCl_2$, 20 mM PIPES pH 6.8). The lysis solution was twice pre-cleared at 500 rcf at 4°C, and the supernatant was divided into three 300 µL aliquots. The aliquots were mixed with 300 µL of PS200, PS200 with 10 µg/mL trypsin (Sigma-Aldrich), and PS200 buffer supplemented with 10 µg/mL trypsin and 0.4% Triton X-100 (Sigma-Aldrich). After 30 min of incubation, the digestion was halted through trichloroacetic acid (TCA, Merck) precipitation and the pellet was washed twice with acetone (Vidrolab 2). The pellets were dissolved in 50 µL of Laemmli buffer. In the case of prApeI protease protection assay, the total lysate (after spheroplast lysis) was divided into a 13,000 x *g* supernatant and pellet fraction after cell debris was pre-cleared at 500 rcf at 4°C. This procedure was used to obtain highly enriched membrane fractions.

3.4.6. CPY colony blot assay

The CPY colony blot assay was done as described (Kojima *et al.*, 2012) with some modifications. Briefly, cells were grown to exponential phase and then diluted to 0.02 OD_{600}, and 3 µL of each dilution was spotted on a YPD plate. After 48 hours of incubation, spots were covered with a 0.45 µm nitrocellulose filter for an additional day. The filter was then removed from the plate, cells were washed off three times with PBS pH 7.4 and the presence of CPY was done by immunoblotting.

3.4.7. Quinacrine staining, mitochondrial mass determination and mitochondrial membrane potential

Vacuolar acidification was measured using quinacrine (Sigma-Aldrich) staining (Seol *et al.*, 2001). Fluorescence was measured on a Becton Dickinson FACS Canto II Analytic Flow cytometer with excitation and emission settings of 405 nm and 515–545 nm, respectively. Mitochondrial mass determination was performed using MitoTracker Green FM (Molecular Probes) according to the manufacturer. Fluorescence was measured by flow cytometry with excitation/emission settings of 488/525 nm (FL-1 channel). The mitochondrial membrane potential was measured as described (Teixeira *et al.*, 2014). Data treatment was performed using the FlowJo software (Tree Star).

3.4.8. Fluorescence microscopy

To monitor GFP-Atg8p processing, cells were grown in SC-medium to the exponential phase and treated with either rapamycin (200 ng/mL, Sigma-Aldrich) or DMSO (vehicle, Sigma-Aldrich) for 3 hours. To analyze vacuolar morphology, cells were grown in YPD to the exponential phase. Cells (20-40 OD_{600} units. mL^{-1} in YPD) were then incubated with 4.8 µM FM4-64 dye (Molecular Probes) for 30 min and washed twice with YPD. Videos were collected in Spinning Disk Confocal System Andor Revolution XD (ANDOR Technology, UK) using iXonEM+DU-897 with 2x port coupler (ANDOR Technology, UK). For mitochondrial morphology analysis, cells carrying pYX222-mtDsRed were grown in SC-medium to the PDS phase. For the mitochondrial fusion during mating, MATa strains carrying mtDsRed (pYX222-mtDsRed) and MATa strains carrying pmtGFP (pVT100U) were grown to the exponential phase in SC-medium lacking histidine and uracil, respectively, pelleted by centrifugation, washed twice with YPD, and ressuspended in 1 mL of YPD medium. Same OD_{600} units of both cells were mixed and collected by centrifugation. Live cells were observed by fluorescence microscopy (AxioImager Z1, Carl Zeiss). Data image stacks were deconvolved by QMLE algorithm of Huygens Professional v3.0.2p1 (Scientific Volume Imaging B.V.). Maximum intensity projection was used to output final images using ImageJ 1.45v software.

3.4.9. Vacuoles isolation

Cells grown to the exponential phase were ressuspended in assay buffer (100 mL of 100 mM Tris-HCl, 10 mM DTT pH 9.5) and incubated at 30ºC for 30 min while shaking at 75 r.p.m. Cells were centrifuged at 4,000 r.p.m. for 5 min, washed with 100 mL of water followed by a wash with 1.1 M sorbitol and ressuspended in 50 mL of 1.1 M sorbitol/20 g cells. The cell wall was enzymatically digested with 1 mL of glusulase (glucuronidase (90,000 units/mL) and sulfatase (19,000 units/mL) (PerkinElmer Life Sciences), 3 mg of zymolyase 20T and 3 mg of Glucanex (Sigma-Aldrich) per 10 mL suspension supplemented with 5 mM DTT. The suspension was incubated for 2.5 h at 30ºC while shaking at 60 r.p.m. Light microscopy was used to evaluate the extent of spheroplast

formation. Spheroplasts were then cooled in ice and each 25 mL of suspension was pipetted on top of a layer of 25 mL of buffer F [7.5% Ficoll (GE Healthcare) containing 1.1 M sorbitol]. The spheroplasts were washed through this Ficoll-sorbitol layer by centrifugation at 4,000 r.p.m. for 20 min at 4ºC. Samples of 20 mL were transferred to a centrifuge tube and overlaid with 10 mL of lysis buffer [10 mM Tris-MES (Sigma-Aldrich), pH 6.9, 12% Ficoll, 0.1 mM $MgCl_2$ and 0.1 mM PMSF]. Centrifugation was performed in a swing-out bucket rotor (Sorvall Ultra Pro 80, Rotor TH641) at 17,000 r.p.m. (50,000 g_{avg}) for 30 min at 4ºC. Vacuoles were collected from the fraction floating on top of the tube, and ressuspended in 10 mL of lysis buffer/ 2-3 mL of crude vacuoles by homogenization with a micropipette. The homogenized crude vacuoles were overlaid in a centrifuge tube with a layer of 10 mL Tris-MES pH 6.9, 8% Ficoll, 0.5 mM $MgCl_2$ and 0.1 mM PMSF and a second layer of 10 mL of the same buffer containing 4% Ficoll instead. Upon centrifugation at 17,000 r.p.m. (50,000 g_{avg}) for 45 min at 4ºC, intact vacuoles were obtained on top of 4% Ficoll solution. Purified vacuoles were collected with a spoon-shaped spatula prewetted in 4% Ficoll buffer.

3.4.10. Co-IP assay

For Isc1p–Dnm1p interaction experiments, a Dnm1p GFP-tagged BY4741 strain carrying FLAG-Isc1p was used. 30 OD_{600} cell equivalents were harvested and ressuspended in 500 µL IP buffer (0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA (Merck), 50 mM Tris, pH 7.4, supplemented with protease and phosphatase inhibitors). Cells were lysed with glass beads and cleared by centrifugation at 13,000 g for 15 min. 250 µL of supernatant was incubated with 25 µL of anti–FLAG (Sigma-Aldrich) for 2 h followed by incubation with protein G Sepharose beads (Protein G Sepharose 4 Fast Flow, GE Healthcare Life Sciences) for 1 h at 4°C. The beads were collected and washed 4 times in washing buffer (1× phosphate-buffered saline [PBS], 2 mM Na_3VO_4, 50 mM KF, 15 mM $Na_2H_2P_2O_7$, 15 mM *p*-nitrophenylphosphate (*p*-NPP), 0.1% Triton X-100 and protease inhibitor cocktail). The bound proteins were released by incubating the beads in 40 µL in Laemmli buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE using 8.5% polyacrylamide gels, and immunodetection was performed with both anti-GFP and anti-FLAG antibodies.

3.4.11. Statistical analysis

Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's t-test. The 0.05 probability level was chosen as the point of statistical significance throughout. Statistical analyses were carried out using GraphPad Prism Software v5.01 (GraphPad Software).

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3.5. Supplementary Information

Table 3.5.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4741	Mata *his3*Δ*1, leu2*Δ*0, met15*Δ*0, ura3*Δ*0*	EUROSCARF
BY4741	Mata *his3*Δ*1, leu2*Δ*0, met15*Δ*0, ura3*Δ*0* [pYX222-mtDsRed, pRS413*TEF*pr-*PEP4*, pRS416-*GFP-ATG8,* pJT30, pDN436 (CPY*-HA), pRS315-HAC1^{i},pRS426-*VAM3,* YEp352-*YKT6*, YEp24-*YPC1*, pJDCEX2]	This study Dilcher *et al.* (2001) Darsow *et al.* (1997) Ellis *et al.* (2004) Escobar-Henriques *et al*. (2006)
BY4742	Matα *his3*Δ*1, leu2*Δ*0, lys2*Δ*0, ura3*Δ*0*	EUROSCARF
VT1	BY4742 *isc1*Δ::*LEU2* [pVT100U]	This study
BY4741 Dnm1-GFP	BY4741 DNM1-GFP::*HisMX6* [pFLAG-*ISC1*]	Life Technologies This study
TYY172	W303-1B *pho13*Δ*::kan pho8*Δ*60*::*HIS3*	Yorimitsu *et al.* (2007)
TYY181	W303-1B *pho13*Δ*::kan pho8*Δ*60*::*HIS3 atg1*Δ*::URA3*	Yorimitsu *et al.* (2007)
TYVT1	W303-1B *pho13*Δ*::kan pho8*Δ*60*::*HIS3 isc1*Δ*::LEU2*	This study
*vac8*Δ	BY4741 *vac8*Δ::*KanMX4*	This study
*isc1*Δ	BY4741 *isc1*Δ::*KanMX4* [pYX222-mtDsRed pRS413*TEF*pr-*PEP4*, pRS416-*GFP-ATG8*, pJT30, pDN436 (CPY*-HA), pRS315-*HAC1^{i}*,pRS426-*VAM3,* YEp352-*YKT6*, YEp24-*YPC1*, pJDCEX2]	EUROSCARF This study

*isc1*Δ Dnm1-GFP	BY4741 DNM1-GFP *isc1*Δ::*LEU2*	This study
*isc1*Δ*vac8*Δ	BY4741 *isc1*Δ::*LEU2 vac8*Δ::*KanMX4*	This study
VT2	*isc1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*tor1*Δ	BY4741 *tor1*Δ::*KanMX4*	Teixeira *et al.* (2014)
VT3	*tor1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*isc1*Δ*tor1*Δ	BY4741 *isc1*Δ::*LEU2 tor1*Δ::*KanMX4*	Teixeira *et al.* (2014)
VT4	BY4741 *isc1*Δ::*URA3 tor1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*sch9*Δ	BY4741 *sch9*Δ::*KanMX4*	EUROSCARF
VT5	*sch9*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*isc1*Δ*sch9*Δ	BY4741 *isc1*Δ::*LEU2 sch9*Δ::*KanMX4*	Teixeira *et al.* (2014)
VT6	BY4741 *isc1*Δ::*URA3 sch9*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*sit4*Δ	BY4741 *sit4*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*]	EUROSCARF This study
VT7	*sit4*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*isc1*Δ*sit4*Δ	BY4741 *isc1*Δ::*LEU2 sit4*Δ::*KanMX4* [pYX222-mtDsRed, pRS416-*GFP-ATG8*]	This study
VT8	BY4741 *isc1*Δ::*URA3 sit4*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study

*hog1*Δ	BY4741 *hog1*Δ::*KanMX4* [pYX222-mtDsRed]	EUROSCARF This study
VT9	*hog1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*isc1*Δ*hog1*Δ	BY4741 *isc1*Δ::*URA3 hog1*Δ::*KanMX4* [pYX222-mtDsRed]	This study
VT10	BY4741 *isc1*Δ::*URA3 hog1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*dnm1*Δ	BY4741 *dnm1*Δ::*KanMX4* [pYX222-mtDsRed]	EUROSCARF This study
VT11	*dnm1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*isc1*Δ*dnm1*Δ	BY4741 *isc1*Δ::*URA3 dnm1*Δ::*KanMX4* [pYX222-mtDsRed]	This study
VT12	BY4741 *isc1*Δ::*URA3 dnm1*Δ::*KanMX4 pho8*Δ::*HPH* [pYX242-mtPho8]	This study
*atg32*Δ	BY4741 *atg32*Δ::*KanMX4* [p*ISC1*]	EUROSCARF This study
*isc1*Δ*atg32*Δ	Matα *isc1*Δ::*LEU2 atg32*Δ::*KanMX4*	This study

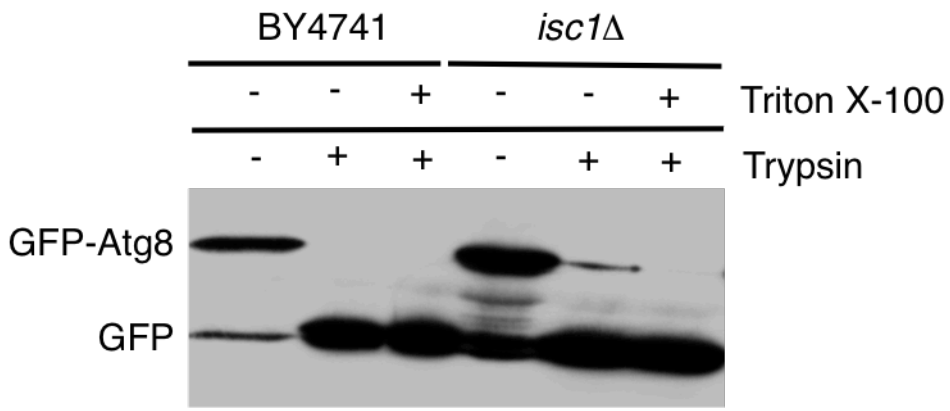


Figure 3.S1. Defective vacuolar proteolysis contributed to reduced autophagic flux in *isc1*Δ cells. BY4741 and *isc1*Δ cells were grown to exponential phase in SC-medium and treated with rapamycin (200 ng/mL) for 3h. Lysed spheroplasts of rapamycin-treated cells were trypsin-digested with and without detergent (Triton X-100). Immunoblots were performed with anti-GFP antibody.

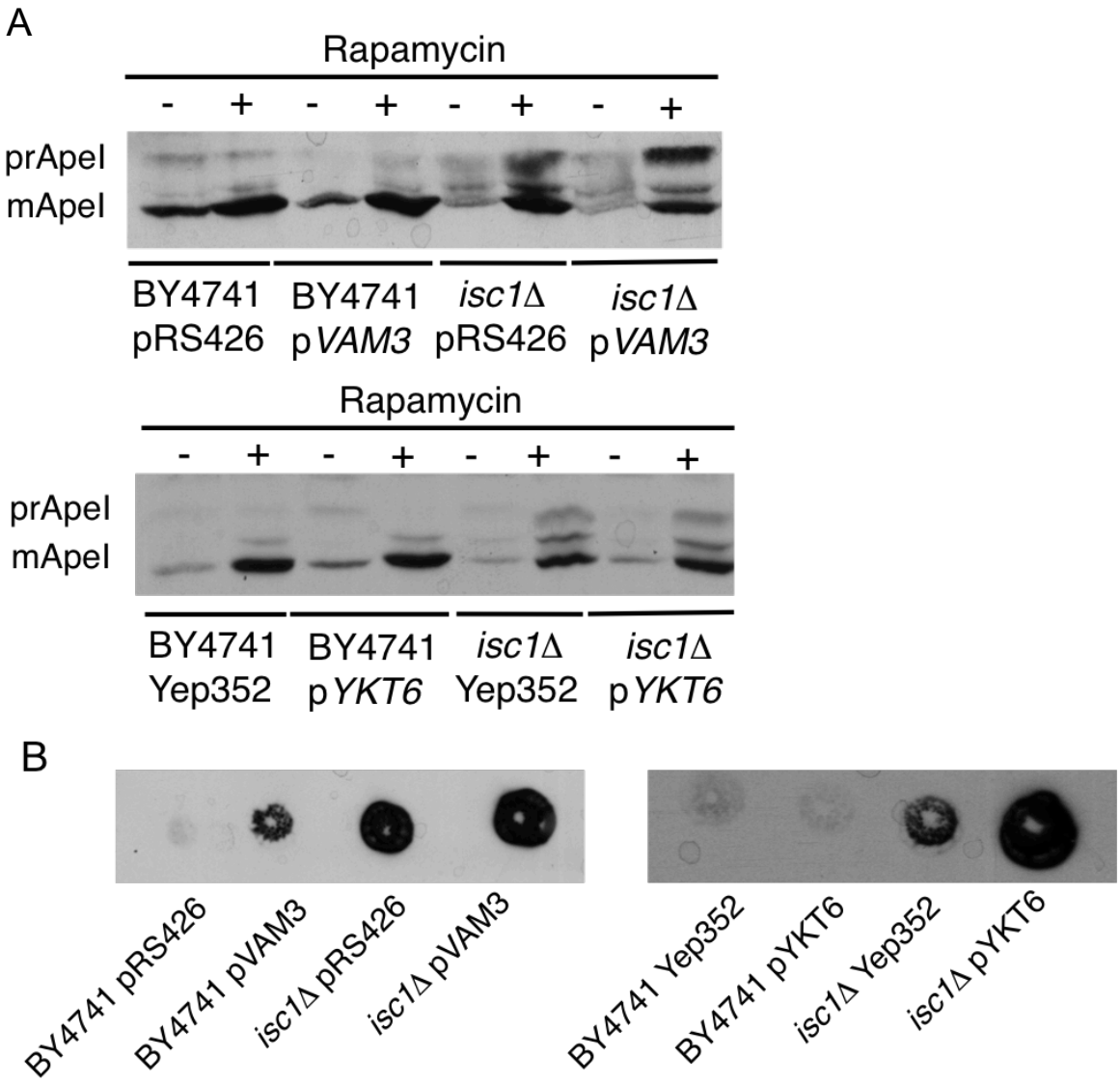


Figure 3.S2. Overexpression of *VAM3* or *YKT6* does not suppress the Cvt pathway and CPY protein sorting defects in *isc1*Δ cells.

A. BY4741 and *isc1*Δ cells overexpressing *VAM3* or *YKT6* were grown to exponential phase in SC-medium and treated with either DMSO (vehicle) or rapamycin (200 ng/mL) for 3h. The processing of the Cvt pathway marker prApeI to mApeI was analyzed by immunoblotting using anti-ApeI. **B.** Cells were grown on SC-medium plates in contact with a nitrocellulose filter. Secreted CPY was immunodetected with anti-CPY.

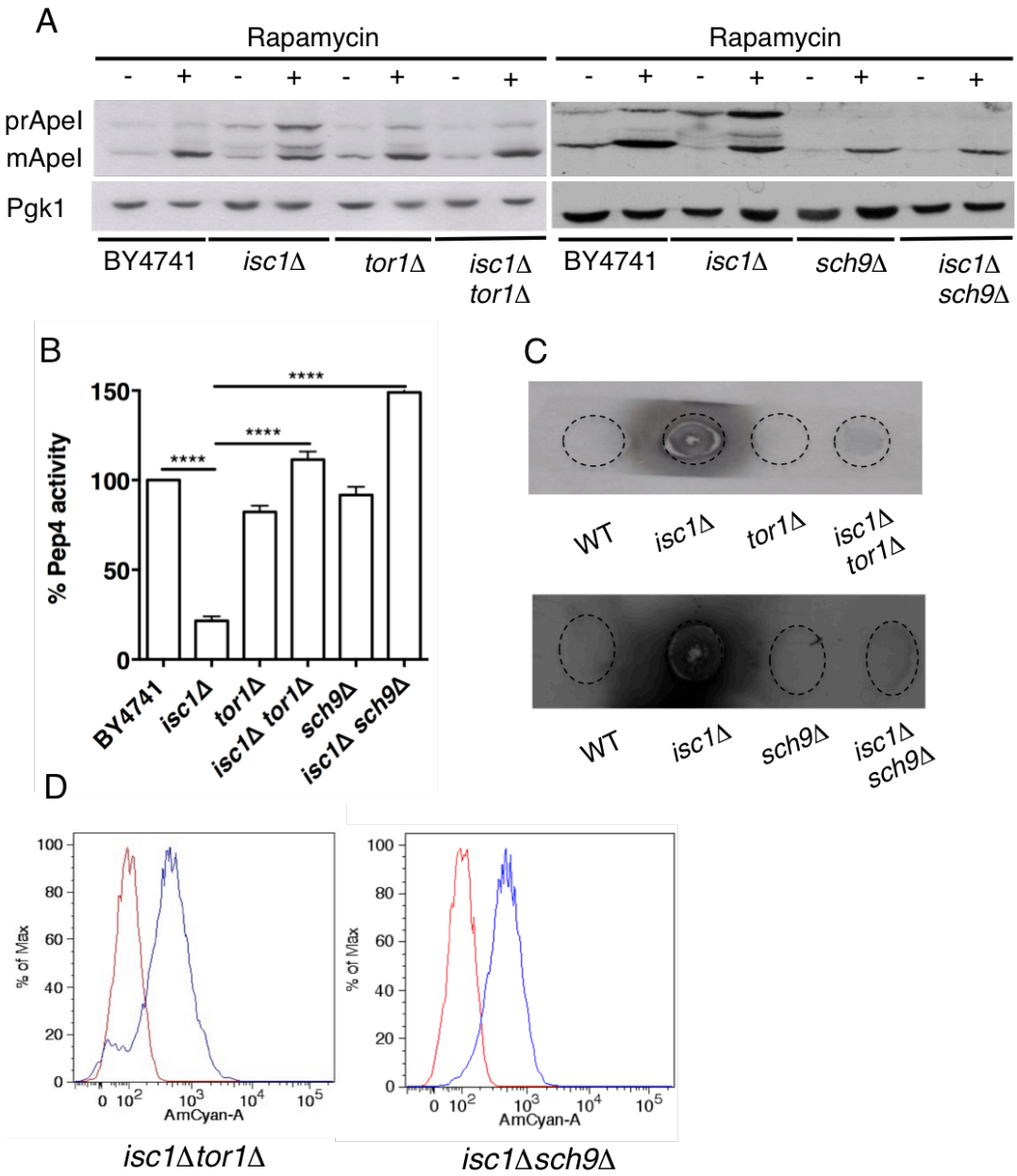


Figure 3.S3. The deletion of *TOR1* and *SCH9* suppressed the Cvt pathway and vacuolar protein sorting defects in *isc1Δ* cells.

A. The processing of the Cvt pathway marker prApeI to mApeI was analyzed by immunoblotting in BY4741, *isc1*Δ, *tor1*Δ, *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells treated with either DMSO (vehicle) or rapamycin (200 ng/mL) for 3h. **B.** Specific Pep4p activity was measured in protein extracts of BY4741, *isc1*Δ, *tor1*Δ, *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells. Values were normalized to parental cells (set as 100%). Values are mean ± SD of at least three independent experiments. ****p<0.0001. **C.** CPY secretion was analyzed in BY4741, *isc1*Δ, *tor1*Δ, *isc1*Δ*tor1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells as described in figure 3.2.D. **D.** Vacuolar acidification of *isc1*Δ*tor1*Δ and *isc1*Δ*sch9*Δ cells was analyzed using quinacrine staining. Unprobed and probed cells are depicted in red and blue, respectively. Representative histograms are shown.

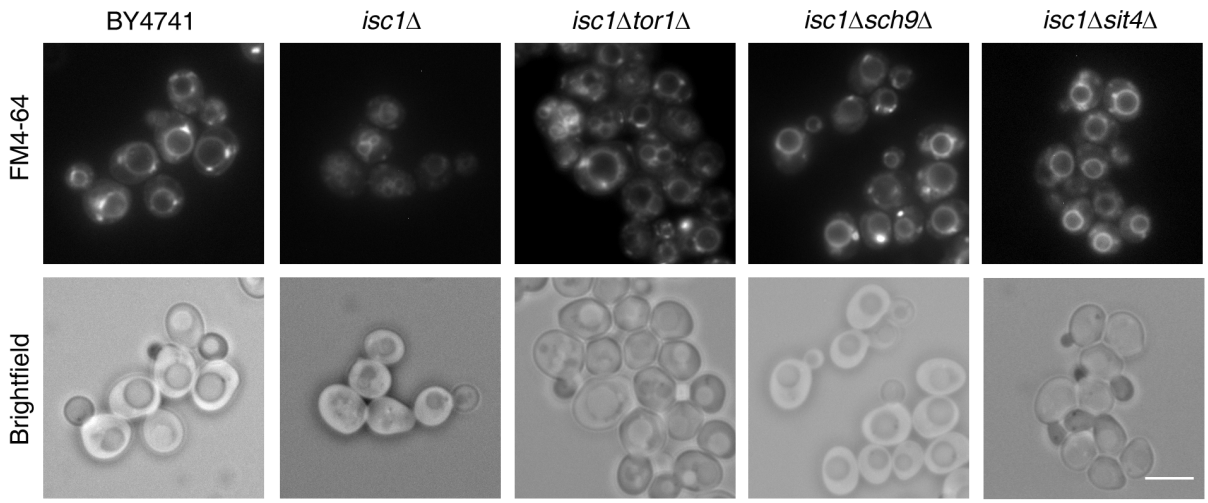


Figure 3.S4. The deletion of *TOR1*, *SCH9* and *SIT4* suppressed vacuolar fragmentation in *isc1*Δ cells. BY4741, *isc1*Δ, *isc1*Δ*tor1*Δ, *isc1*Δ*sch9*Δ and *isc1*Δ*sit4*Δ cells were grown to exponential phase and incubated with FM 4-64 for 30 min. Vacuoles were observed by fluorescence microscopy. Scale bar: 5 μm.

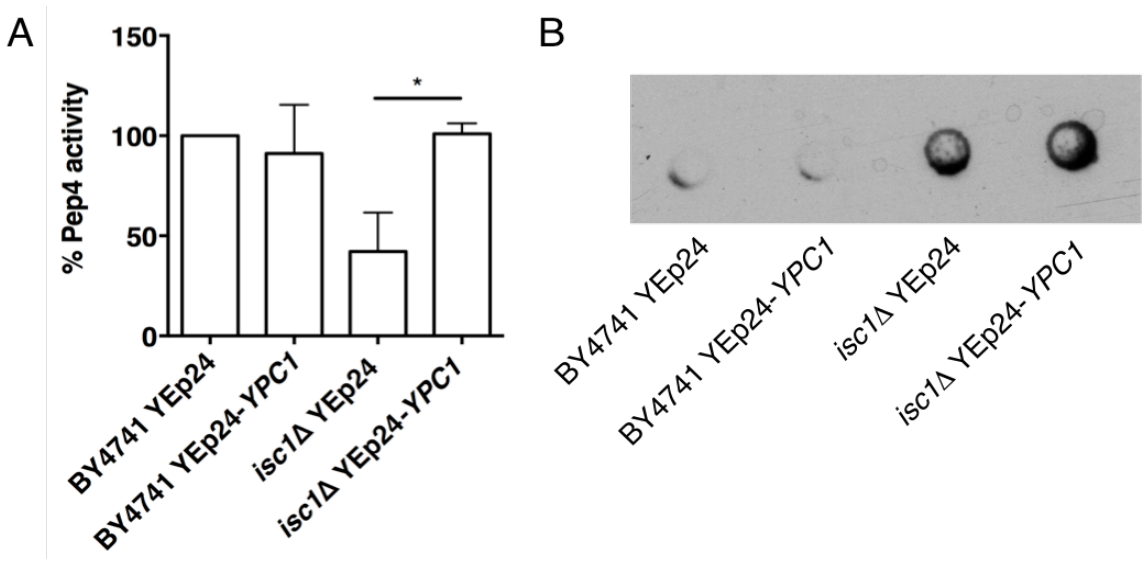


Figure 3.S5. Ectopic overexpression of *YPC1* abolishes Pep4p-dependent vacuolar proteolysis but not the vacuolar protein sorting defect in *isc1*Δ cells. A. BY4741 and *isc1*Δ cells carrying either YEp24 (empty vector) or overexpressing *YPC1* were grown to exponential phase in SC-medium. Specific Pep4p activity was measured and values were normalized to parental cells (set as 100%). Values are mean ± SD of at least three independent experiments. *p<0.05. **B.** BY4741 and *isc1*Δ cells carrying either YEp24 (empty vector) or overexpressing *YPC1* were grown on SC-medium plates in contact with a nitrocellulose filter. Secreted CPY was immunodetected with anti-CPY.

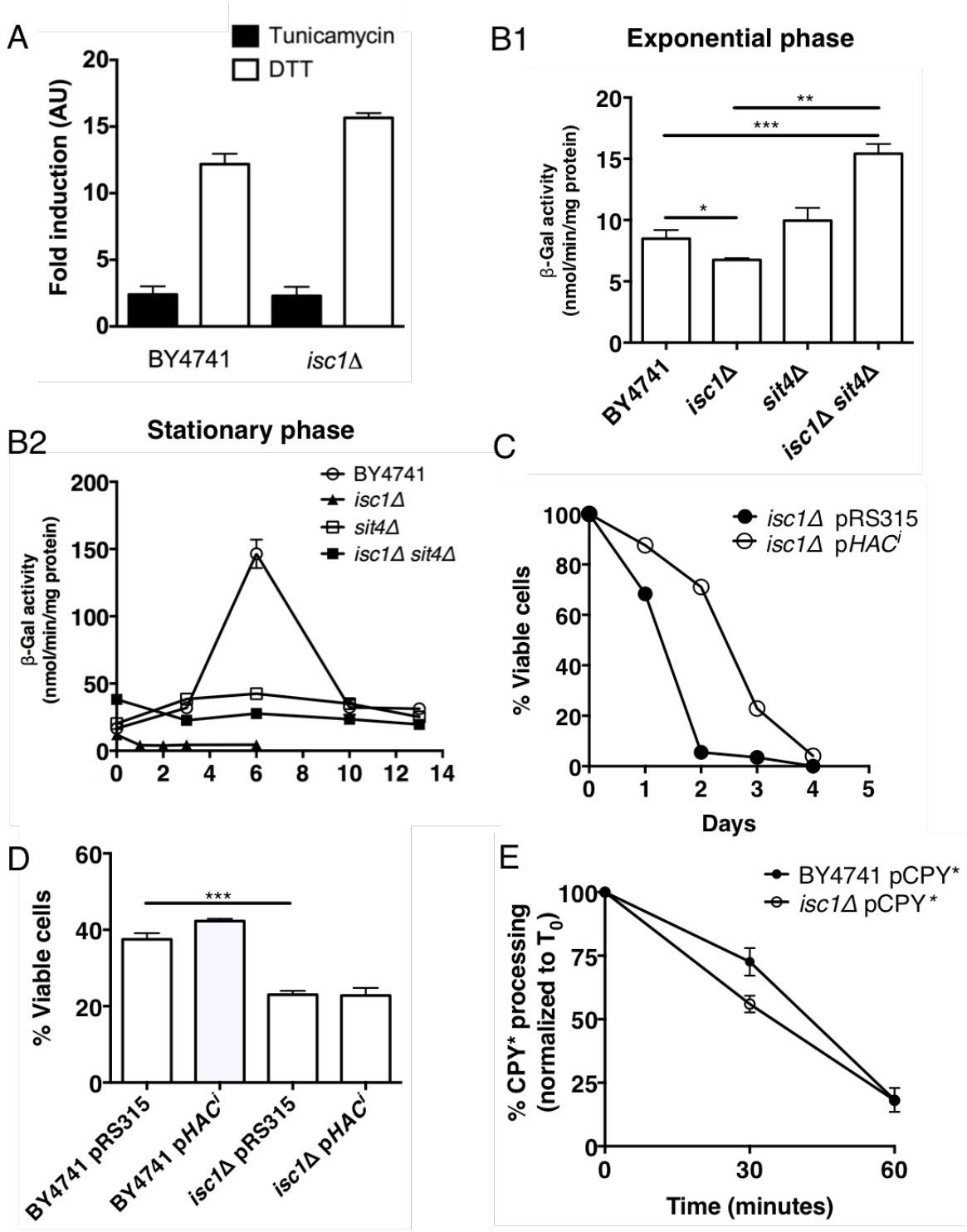


Figure 3.S6. The UPR response is downregulated by a Sit4p-dependent mechanism in *isc1*Δ cells.

A. BY4741 and *isc1*Δ cells carrying an UPRE-lacZ reporter (pJT30) were grown in SC-medium to the exponential phase and treated with either tunicamycin (2 μg/mL, vehicle DMSO) or DTT (6 mM, vehicle water) for 1 h. β-galactosidase activity was determined. Values represent the fold induction between untreated and treated cells and are means ± SD of at least three independent experiments. **B.** BY4741, *isc1*Δ, *sit4*Δ and

*isc1*Δ*sit4*Δcells carrying an UPRE-LacZ reporter (pJT30) were grown in SC-medium to the exponential (B1) or stationary phase (B2) and kept in the medium overtime. β-galactosidase activity was determined. Values are means ± SD of at least three independent experiments. ***p<0.001; **p<0.01; *p<0.05. **C.** Isc1p-deficient cells carrying the empty vector (pRS315) and expressing the intron-less form of the *HAC1* gene (pHAC^{i}) were grown to PDS phase in SC-medium and kept in the medium overtime. The viability was expressed as the percentage of the colony-forming units as compared to time T_0. **D.** BY4741 and *isc1*Δ cells carrying pRS315 or p*HAC*^{i} were grown in SC-medium to the exponential phase and exposed to 1.5 mM H_2O_2 for 60 min. Cell viability was expressed as the percentage of the colony-forming units (treated cells versus untreated cells). Values are mean ± SD of at least three independent experiments. ***p<0.001. **E.** BY4741 and *isc1*Δ cells carrying CPY*–HA were treated with cycloheximide (to block protein synthesis) for 1h and aliquots were collected at the indicated times. Immunoblotting was performed using anti-HA or anti-actin as primary antibodies. The CPY*-HA band intensities were normalized to actin bands. Data was expressed as the percentage relative to time zero.

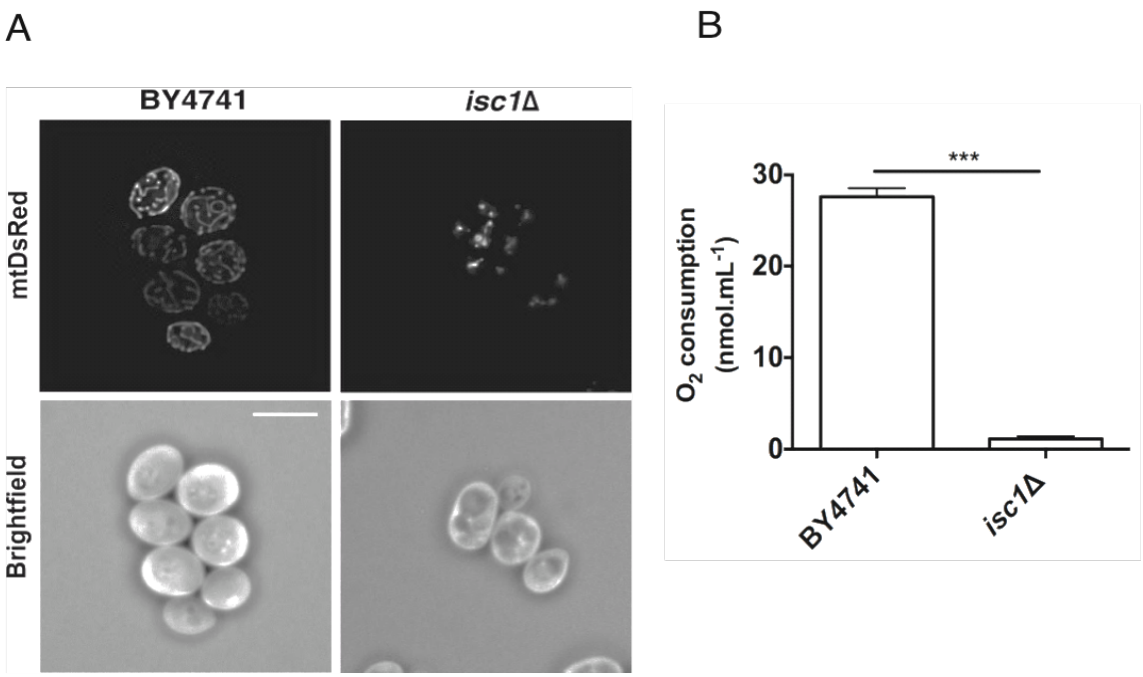


Figure 3.S7. Isc1p-deficient cells exhibit mitochondrial fragmentation and reduced oxygen consumption in lactate-growing conditions. BY4741 and *isc1*Δ cells carrying mtDsRed were grown in SC-glucose (OD_{600}=0.1) and shifted to SC-lactate medium for 48 h. The mitochondrial network was analyzed by fluorescence microscopy (scale bar: 5 μm) (A) and oxygen consumption rate was measured (B).

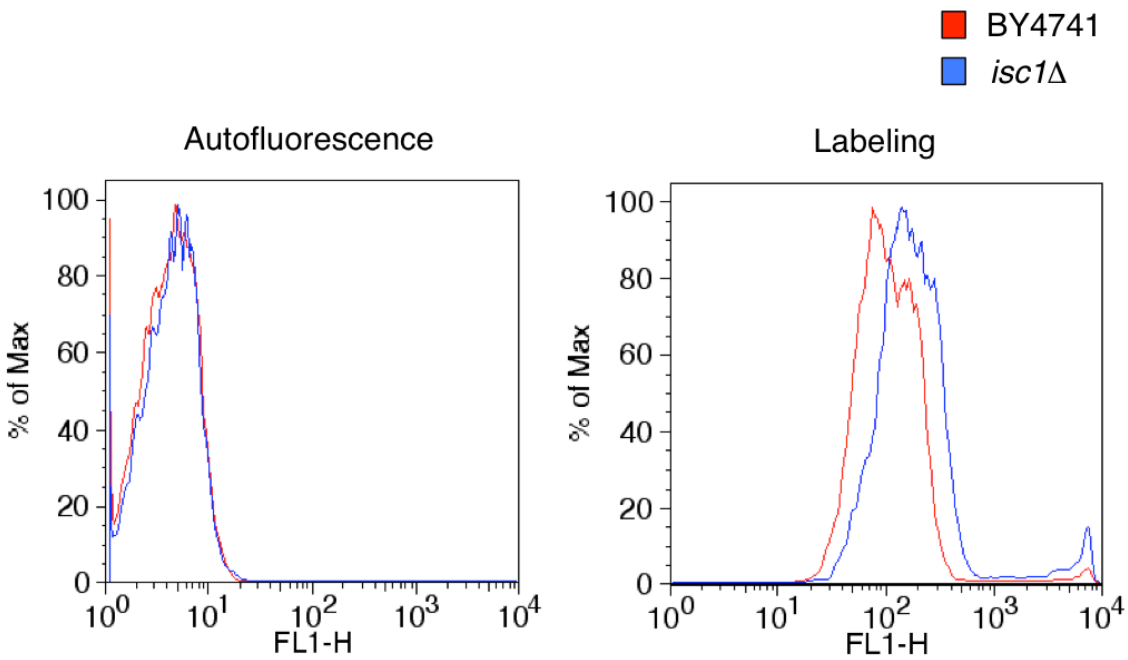


Figure 3.S8. The mitochondrial mass is increased in *isc1*Δ cells at PDS phase. BY4741 and *isc1*Δ cells were grown in SC-medium to PDS phase and ressuspended in 10 mM HEPES buffer, pH 7.4, containing 5% glucose (10^6 cells/mL). The probe MitoTracker Green FM was added to a final concentration of 100 nM and cells were incubated at 26ºC for 30 minutes. Representative histograms are shown.

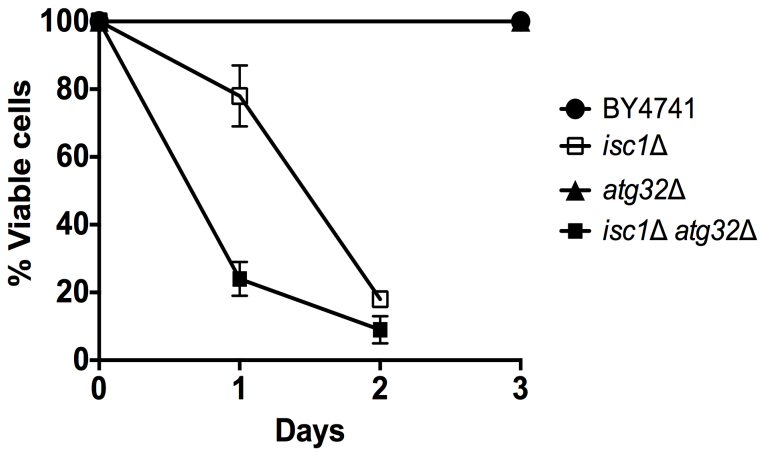


Figure 3.S9. The deletion of *ATG32* shortened the CLS of *isc1*Δ cells. Cells were grown in SC-glucose to the PDS phase (T_0) and kept in the medium at 26°C. The viability was determined as the percentage of the colony-forming units at time T in relation to T_0. Values are mean ± SD of at least three independent experiments.

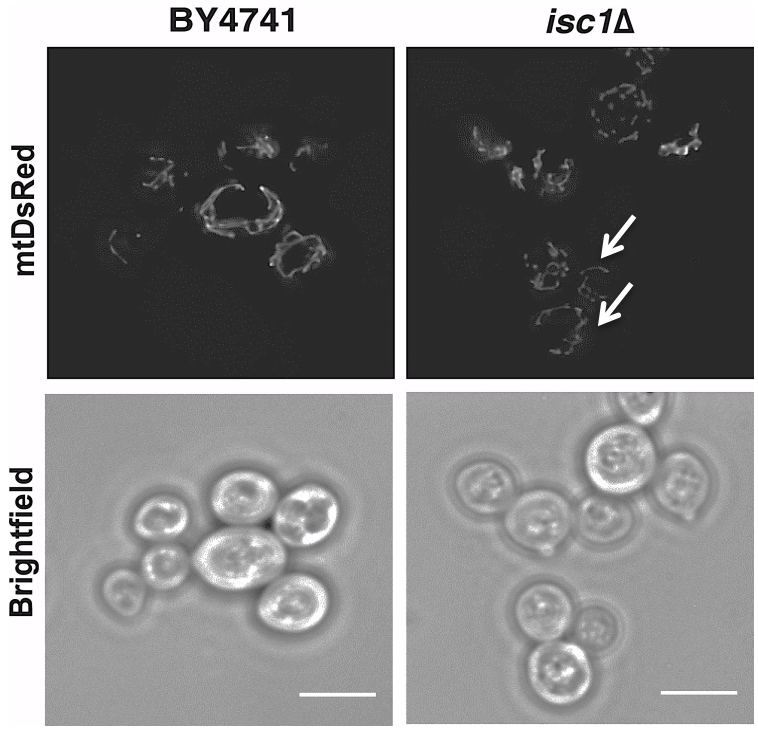


Figure 3.S10. Overexpression of *FZO1* partially abolishes mitochondrial fragmentation of *isc1*Δ cells. BY4741 and *isc1*Δ cells carrying FLAG-*FZO1* (pJDCEX2) and mtDsRed were grown in SC-glucose lacking leucine and histidine in the presence of 50 mM $CuSO_4$ (to induce *FZO1* expression) and the mitochondrial network was observed by fluorescence microscopy. Scale bar: 5 μm.

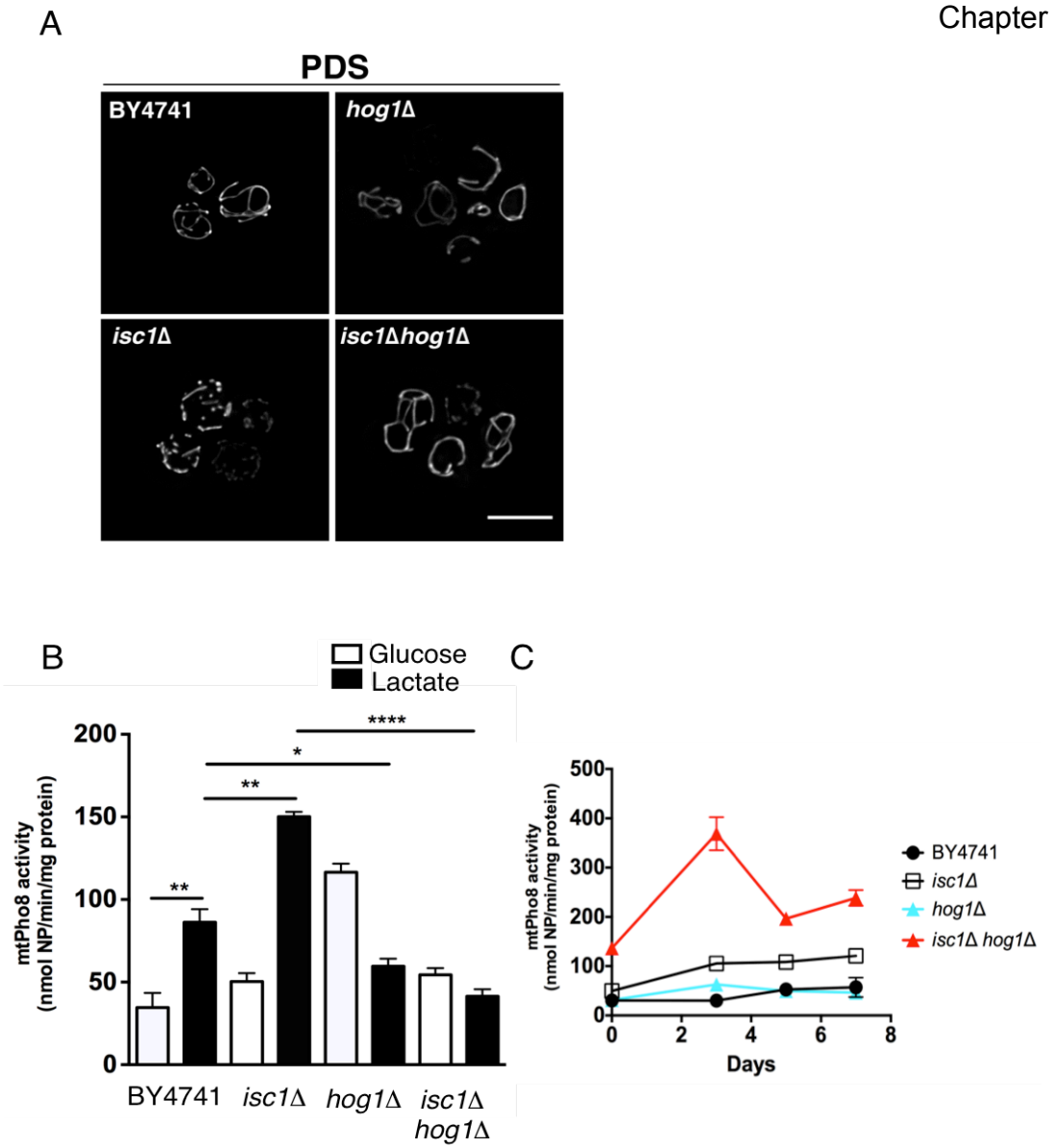


Figure 3.S11. The deletion of *HOG1* suppressed mitochondrial fragmentation and this was correlated with lower induction of mitophagy in *isc1Δ* cells in lactate-growing conditions.

A. BY4741, *isc1*Δ, *hog1*Δ and *isc1*Δ*hog1*Δ cells carrying mtDsRed were grown to PDS phase and mitochondrial network was analyzed by fluorescence microscopy. Representative images are shown. Scale bar: 5 μm. **B.** BY4741, *isc1*Δ, *hog1*Δ and *isc1*Δ*hog1*Δ cells carrying mtPho8 were shifted from glucose (OD_{600}=0.1) to lactate medium for 48 h. Mitophagy activity was measured by the alkaline phosphatase assay in glucose (open bars) or lactate-growing cells (closed bars) after 48 hours. Values are mean ± SD of at least three independent experiments. ****p<0.0001; **p<0.01; *p<0.05.

C. BY4741, *isc1*Δ, *hog1*Δ and *isc1*Δ*hog1*Δ cells carrying mtPho8 were grown in SC-glucose to PDS phase, washed twice, and incubated in water during aging. Mitophagy activity was measured as described in figure 3.4.A. Values are mean ± SD of at least three independent experiments.

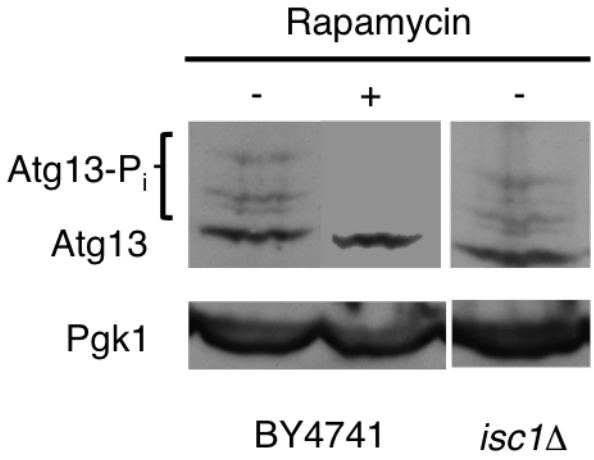


Figure 3.S12. Atg13p phosphorylation is similar in parental and *isc1*Δ cells in vegetative conditions. BY4741 and *isc1*Δ cells were grown to exponential phase in SC-medium. The phosphorylation status of endogenous Atg13p was analyzed by immunoblotting using anti-Atg13p antibody. The dephosphorylated form of Atg13p was discriminated after treatment with rapamycin (200 ng/mL) for 3 h.

CHAPTER IV

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein

Ceramide-activated protein phosphatase Sit4p deficiency extends lifespan in yeast by regulating sphingolipid signalling in a Snf1p-dependent manner

Vitor Teixeira, Rita Vilaça, Paulo Oliveira, Pedro Moradas-Ferreira and Vítor Costa

Keywords: Aging; ceramide; Long chain base; myriocin; Sit4p; sphingolipids; TORC2; Ypk1p

Abstract

Sphingolipids are essential components of membranes and play an important role in several biological functions, namely cell growth, stress response and longevity. In this study, we show that cells lacking Sit4p, a ceramide-activated protein phosphatase orthologue to human protein phosphatase PP6, are sensitive to myriocin and phytosphingosine, indicating alterations in sphingolipid homeostasis. We also demonstrate that Sit4p regulates sphingolipids biosynthetic pathway by a transcriptional regulatory mechanism involving the ceramide synthase *LAG1* and the phytoceramidase *YPC1*. Importantly, myriocin sensitivity is correlated with alterations in Ypk1p-Orm signalling in *sit4*Δ cells. Moreover, the LCBs responsive Pkh1/2p-Sch9p pathway is downregulated in *sit4*Δ cells by a Snf1p-dependent mechanism, a phenotype that contributes to improved mitochondrial function and lifespan extension displayed by *sit4*Δ cells. These results provide evidence that Sit4p is not only an effector but also a key regulator of sphingolipid metabolism and integrates signals derived from TORC1-mediated nutrient sensing with sphingolipid metabolism to regulate longevity in yeast.

4.1. Introduction

Ceramides and sphingoid long-chain bases (LCBs) are sphingolipid metabolites that play important roles in cell signalling and intracellular trafficking (Cowart *et al.*, 2006, Hannun & Obeid, 2008, Liu *et al.*, 2013, Rego *et al.*, 2014). Conserved biosynthetic pathways in yeast are responsible for the formation of bioactive metabolites, such as LCBs, which are represented by phytosphingosine (PHS) and dihydrosphingosine (DHS), the phosphorylated forms of LCBs (LCBPs), namely DHS-1-P and PHS-1-P, and ceramide species, represented by dihydroceramides, phytoceramides and α-hydroxylated phytoceramides (Hannun & Obeid, 2008, Rego *et al.*, 2014). In yeast, they play a crucial role in the regulation of cell growth, endocytosis, actin cytoskeleton organization, protein trafficking, cell wall integrity, nutrient uptake, stress response and importantly longevity (Cowart *et al.*, 2006, Dickson, 2008, Liu *et al.*, 2013, Rego *et al.*, 2014).

Several studies in mammalian and yeast systems have unraveled an interplay between sphingolipid dynamics and the Target of Rapamycin (TOR) signalling pathway to regulate aging. The TOR complex 1 (TORC1) and the TOR complex 2 (TORC2) play a wide spectrum of cellular activities that are crucial for proper regulation of spatial and temporal aspects of cell growth and recently both branches were implicated in the regulation of sphingolipid metabolism (de Virgilio & Loewith, 2006, Evans *et al.*, 2011, Kim & Guan, 2011, Loewith & Hall, 2011, Laplante & Sabatini, 2012, Johnson *et al.*, 2013). Both are involved in the regulation of sphingolipid homeostasis by modulating the activity of Orm1p and Orm2p, two evolutionary conserved proteins located in the endoplasmic reticulum (ER) (Roelants *et al.*, 2011, Liu *et al.*, 2012, Shimobayashi *et al.*, 2013). In particular, TORC1 negatively controls the synthesis of complex sphingolipids from ceramide by inhibiting its downstream effector, the PP2A-like protein phosphatase Sit4p. As a result, the Npr1p kinase is no longer activated and Orm1/2p are not phosphorylated and activated, impeding the flux of ceramide to drive the synthesis of complex sphingolipids (Shimobayashi *et al.*, 2013).

TORC2, on the other hand, stimulates the *de novo* biosynthesis of sphingolipids upon sphingolipid depletion (e.g., by myriocin treatment) via phosphorylation and activation of Ypk1p, which then phosphorylates and

inactivates Orm1/2p (Roelants *et al.*, 2011, Berchtold *et al.*, 2012). Since Orm1/2p bind to and inhibit the serine palmitoyl-coenzyme A transferase (SPT) (Breslow *et al.*, 2010), which catalyzes the first step in sphingolipid biosynthesis, their inactivation by Ypk1p-dependent phosphorylation alleviates SPT inhibition and boosts sphingolipid production (Breslow *et al.*, 2010, Roelants *et al.*, 2011, Berchtold *et al.*, 2012). In addition, TORC2 stimulates ceramide synthase by a Ypk2p-dependent mechanism (Ypk2p is a paralog of Ypk1p), which is antagonized by calcineurin (Aronova *et al.*, 2008). It also inhibits Isc1p, an homologue of mammalian neutral sphingolmyelinase 2 (nMase2), via modulation of Slm1/2p (Tabuchi *et al.*, 2006). The phosphorylation of Slm1/2p by TORC2 promotes their recruitment to the plasma membrane, where they play an essential role in the regulation of Ypk1p activation by increasing the phosphorylation efficiency of Ypk1p by TORC2 (at the HM site) and by Pkh1p kinase (at the A-loop) (Tabuchi *et al.*, 2006, Berchtold *et al.*, 2012, Niles & Powers, 2012). Thus, Slm1/2p are involved in the regulation of both ceramide biosynthesis, via the TORC2-Ypk1p-SPT-ceramide synthase cascade, and ceramide generation by hydrolysis of complex sphingolipids mediated by Isc1p. Importantly, TORC1 regulates Orm phosphorylation by molecular mechanisms dissimilar from TORC2, as Orm phosphorylation sites are distinctly regulated by TORC1 and TORC2 (Huber *et al.*, 2009, Soulard *et al.*, 2010).

Apart from sensing nutrient signalling (aminoacids), early studies have shown that the TORC1 downstream effector and S6K1 homologue, Sch9p, is also responsive to LCBs (PHS) (Liu *et al.*, 2005). Like Ypk1p, Sch9p is phosphorylated by Pkh1/2p at its PDK1 site located in the activation loop (Roelants *et al.*, 2004, Urban *et al.*, 2007), which is indispensable for proper activity. The dual regulation of Sch9p by TORC1 and Pkh1p is crucial for proper integration of nutrient and sphingolipid signalling impacting on cell growth and survival (Huang *et al.*, 2012, Huang *et al.*, 2013). In fact, reducing the rate of the sphingolipid biosynthetic pathway by myriocin treatment leads to chronological lifespan (CLS) extension and improvement of stress response by downregulating the Pkh1/2p-Sch9p pathway, although Sch9p-independent mechanisms are also implicated (Huang *et al.*, 2012). More recently, Sch9p was shown to be a key regulator of sphingolipid metabolism as the LCBs/ceramide rheostat is altered in *sch9*Δ mutants in favor of increased survival (Swinnen *et al.*, 2014).

On the other hand, the deletion of both *TOR1* and *SCH9* also improves mitochondrial function and oxidative stress response by an intrinsic mechanism involving enhanced mitochondrial membrane potential and superoxide production, which pre-conditions cells to better survive at the stationary phase and extends CLS in yeast (Bonawitz *et al.*, 2007, Wei *et al.*, 2008, Wei *et al.*, 2009, Pan *et al.*, 2011). Such mechanisms are highly conserved from yeast to mammals (Swinnen *et al.*, 2013). More recently, we have proposed that Sch9p also integrates nutrient signals from TORC1 with Isc1p-driven ceramide signalling in the regulation of mitochondrial function, oxidative stress resistance and CLS in yeast (Teixeira *et al.*, 2014).

The TORC1 downstream effector Sit4p is the catalytic subunit of the ceramide-activated protein phosphatase (CAPP), a heterotrimeric complex that also includes Tpd3p and Cdc55p as regulatory subunits (Nickels & Broach, 1996). Sit4p was also shown to associate with a set of proteins collectively denominated SIT4-associated proteins (SAP), which positively regulates Sit4p activity (Luke *et al.*, 1996). Sit4p is a serine-threonine protein phosphatase related to type 2A family of protein phosphatases (PP2A), with high homology to human protein phosphatase 6 (Bastians & Ponstingl, 1996, Morales-Johansson *et al.*, 2009). Sit4p regulates several biological processes, such as the cell wall integrity (CWI) pathway, cytoskeleton organization and ribosomal gene expression (Angeles de la Torre-Ruiz *et al.*, 2002). It also modulates the cell cycle by regulating the Swi4p-mediated transcription of G_1 cyclin genes (Fernandez-Sarabia *et al.*, 1992, Di Como *et al.*, 1995, Angeles de la Torre-Ruiz *et al.*, 2002), ion tolerance (Masuda *et al.*, 2000), multidrug resistance (Chen, 2001), the ubiquitin-proteasome system (Singer *et al.*, 2003), endocytosis (McCourt *et al.*, 2009) and TORC1 readouts, namely on nutrient signalling (Rohde *et al.*, 2004, Tate *et al.*, 2006, Tate *et al.*, 2009) and sphingolipid metabolism (Barbosa *et al.*, 2011, Shimobayashi *et al.*, 2013). In response to nutrient signals, TORC1 modulates the interaction of Sit4p with its essential effector Tap42p and thereby controls Gcn2p-regulated translation, expression of the nitrogen catabolite-repressed (NCR) and retrograde response genes (Beck & Hall, 1999, Bertram *et al.*, 2000, Torres *et al.*, 2002, Kubota *et al.*, 2003, Rohde *et al.*, 2004). Importantly, Sit4p is also involved in the regulation of mitochondrial function. In fact, *sit4*Δ cells present a growth defect on respiratory substrates by shunting the carbohydrate metabolism flux into

gluconeogenesis and glycogen storage, therefore depleting the Krebs cycle intermediates (Jablonka *et al.*, 2006). In addition, Sit4p participates in the catabolic derepression of mitochondria. In glucose-growing cells, both Mig1p and hexocinase Hxk2p are dephosphorylated by the Reg1p-Glc7p protein phosphatase complex and form a complex in the nucleus to repress the expression of genes associated with growth on non-fermentable carbon sources (Ahuatzi *et al.*, 2004, Ahuatzi *et al.*, 2007). The interaction between Hxk2p and Mig1p under repressing conditions inhibits Mig1p phosphorylation at Ser311 by the Snf1p protein kinase, a homologue of mammalian AMPK, and its export from the nucleus into the cytosol in a Msn5p-dependent manner (Ahuatzi *et al.*, 2007). Under these conditions, Snf1p is dephosphorylated and inhibited by Reg1p-Glc7p and Sit4p protein phosphatases (Ruiz *et al.*, 2011).

We have previously reported that cells lacking Isc1p exhibit mitochondrial dysfunctions, oxidative stress sensitivity and premature aging associated with the activation of Sit4p in response to increased levels of very long chain ceramide species during aging (Almeida *et al.*, 2008, Barbosa *et al.*, 2011). Although Sit4p functions downstream of Isc1p (Barbosa *et al.*, 2011), the phosphatase is also downregulated by TORC1 (Di Como & Arndt, 1996, Jacinto *et al.*, 2001, de Virgilio & Loewith, 2006) and Sit4p controls the turnover of complex sphingolipids in response to TORC1 in a Npr1p-dependent manner (Shimobayashi *et al.*, 2013). In addition, a reduction in Orm1p phosphorylation was observed in *sit4*Δ cells at basal conditions and in response to myriocin, indicating that Sit4p is also an effector of sphingolipid signalling (Liu *et al.*, 2012). Notably, *SIT4* (Barbosa *et al.*, 2011) and *TOR1* (Bonawitz *et al.*, 2007, Pan *et al.*, 2011) deletion extends lifespan by boosting mitochondrial function and stress responses in yeast.

How Sit4p integrates nutrient and sphingolipid signalling to regulate lifespan remains poorly understood. This works aimed to establish a functional association between Sit4p and sphingolipid metabolism and evaluate the impact on cell survival. Here, we show that Sit4p regulates the sphingolipid biosynthetic pathway by a transcriptional regulatory mechanism involving *LAG1* (encoding for ceramide synthase) and *YPC1* (encoding for phytoceramidase) expression. Importantly, decreased Orm phosphorylation (Liu *et al.*, 2012) and ceramide levels (Vilaça, 2014) in *sit4*Δ cells correlate with alterations in Ypk1p-Pkh1p-Orm signalling. Moreover, the activation of the Pkh1p-Sch9p pathway is mostly abolished in *sit4*Δ

cells by a Snf1p-dependent mechanism and this is associated with improved mitochondrial function, oxidative stress resistance and extended CLS displayed by Sit4p-deficient cells.

4.2. Results

4.2.1. Sit4p-deficient cells display alterations in sphingolipid metabolism

To investigate the role of Sit4p in the modulation of sphingolipid biosynthesis, we have analyzed the sensitivity of *sit4*Δ cells to myriocin, an inhibitor of SPT that catalyzes the condensation of palmitoyl-CoA and serine in the *de novo* biosynthetic pathway (Hanada *et al.*, 2000). The analysis was also performed with different selection markers used for the deletion of *SIT4* to exclude possible marker effects. The results show that *sit4*Δ cells exhibited myriocin sensitivity and are more sensitive to the growth-inhibitory effect of PHS (Figure 4.1). Overall, the results suggest that Sit4p fulfills a regulatory role within the sphingolipid biosynthetic pathway.

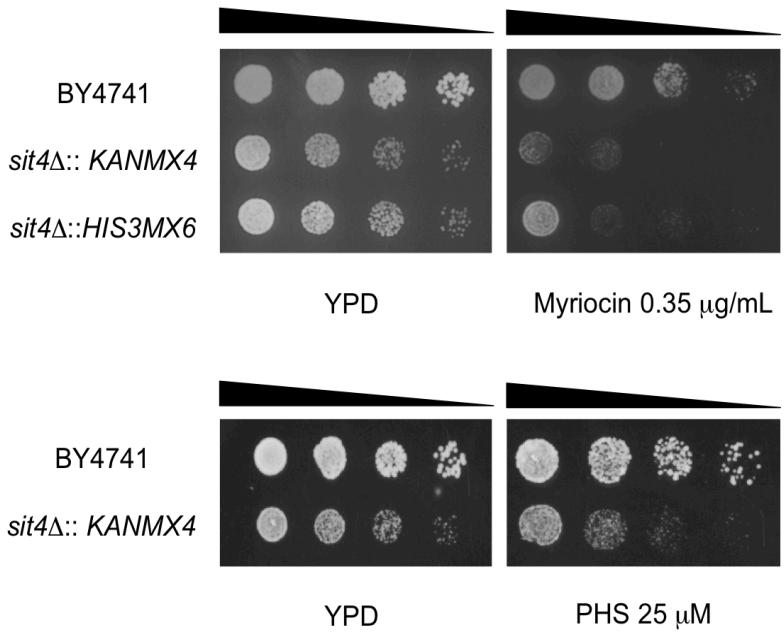


Figure 4.1. Sit4p-deficient cells are sensitive to myriocin and phytosphingosine (PHS). BY4741 and *sit4*Δ cells were grown in SC medium to exponential phase, diluted to OD_{600}=0.1 and spotted in five-dilution series onto YPD plates supplemented with either ethanol 95% and DMSO (vehicle for myriocin and PHS treatment, respectively) or myriocin (0.35 μg/mL) and PHS (25 μM). A representative image out of three is shown.

Consistent with this hypothesis, Sit4p-deficient cells exhibit significant changes in the levels of sphingolipids (Vilaça, 2014). The levels of C14-, C16-C18-, C20-, C22-, C24- and C26-phytoceramides and C14- and C16 dihydroceramides were increased whereas the levels of C18-, C20- and C22-dihydroceramide were decreased in *sit4*Δ cells, indicating some substrate specificity regarding the sphingoid base. Overall, total ceramide content was decreased in *sit4*Δ cells. Moreover, the PHS levels remained unaltered whereas the levels of DHS were increased in *sit4*Δ cells (Vilaça, 2014). The higher levels of LCBs may explain the increased sensitivity to PHS displayed by the mutant strain. Furthermore, Sit4p-deficient cells present higher levels of PHS-1-P and DHS-1-P and lower LCBs/LCBPs ratios compared to parental cells (Vilaça, 2014), suggesting that LCBs are preferentially shunted towards LCBPs formation. These results exclude the possibility that defective ceramide production results from limiting amounts of LCBs.

High levels of LCBPs have been associated with cell growth inhibition (Kim *et al.*, 2000, Zhang *et al.*, 2001). However, the accumulation of LCBPs does not contribute to reduced growth rate of *sit4*Δ cells since the deletion of *LCB4*, which encodes for the major LCB kinase responsible for the synthesis of LCBPs, did not improve cell growth (Figure 4.2).

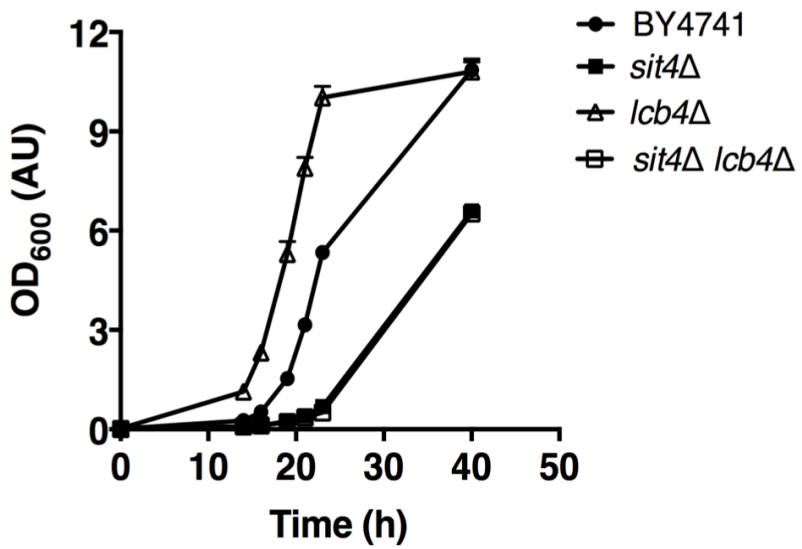


Figure 4.2. The deletion of *LCB4* does not suppress the growth defect of *sit4*Δ cells. Growth curves of BY4741, *sit4*Δ, *lcb4*Δ and *sit4*Δ*lcb4*Δ cells grown in SC-glucose medium. Values are mean ± SD of three independent experiments.

4.2.2. Sit4p controls ceramide levels via transcriptional regulation mechanisms

Sit4p deficiency alters the LCBPs/ceramide rheostat and favors the production of phytoceramide and α-hydroxylated phytoceramides and the accumulation of LCBPs (Vilaça, 2014). Thus, we have also considered that Sit4p could play a regulatory role on sphingolipid biosynthesis and turnover. Since Sit4p is involved in transcriptional programs (Di Como & Arndt, 1996, Crespo *et al.*, 2002, Shirra *et al.*, 2005), we have analyzed the expression of *YDC1* and *YPC1* genes (encoding for ceramidases) and *LAG1* and *LAC1* (encoding for ceramide synthase) in parental and *sit4*Δ cells transformed with LacZ reporters under the control of the promoter of each gene (Swinnen *et al.*, 2014). The analysis of β-galactosidase activity showed that *YPC1* expression decreased and *LAG1* expression increased whereas *YDC1* and *LAC1* expression remained unchanged in *sit4*Δ cells (Figure 4.3). Our results point to the existence of a transcriptional regulation mechanism in which Sit4p acts to promote the induction of *YPC1* ceramidase and the repression of *LAG1* expression in the sphingolipid biosynthetic pathway.

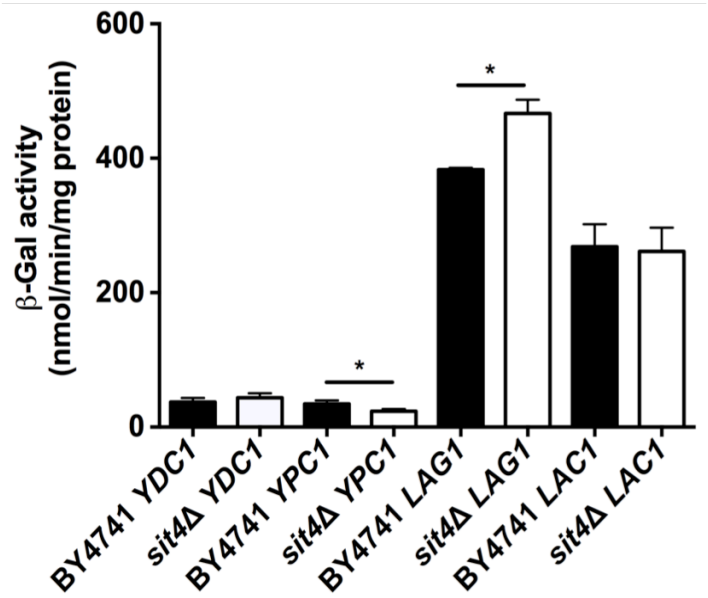


Figure 4.3. The deletion of *SIT4* decreases the expression of ceramidase *YPC1* and increases ceramide synthase *LAG1* expression in yeast. BY4741 and *sit4*Δ cells carrying LacZ reporter fusions with the promoters of *LAG1*, *LAC1*, *YDC1* or *YPC1* in the

multicopy plasmid YEp357 were grown in SC-medium to exponential phase. β-galactosidase activity was determined. Values are means ± SD of at least three independent experiments. *p<0.05.

4.2.3. Snf1p regulates the TORC2-Ypk1p and the Pkh1p-Sch9p signalling pathways in *sit4*Δ cells

Accumulating evidence has demonstrated a functional convergence between the activities of Snf1p and Sit4p in yeast, particularly on reciprocal effects on transcription (Shirra *et al.*, 2005). Ruiz *et al.* reported that Sit4p contributes to the inactivation of Snf1p by dephosphorylating Thr210 (Ruiz *et al.*, 2011). To assess the role of Snf1p in *sit4*Δ phenotypes, we have firstly analyzed myriocin resistance of *sit4*Δ*snf1*Δ cells. Remarkably, the deletion of *SNF1* weakly conferred myriocin resistance to parental cells (Yucel & Ulgen, 2013) and abolished myriocin sensitivity of *sit4*Δ cells (Figure 4.4).

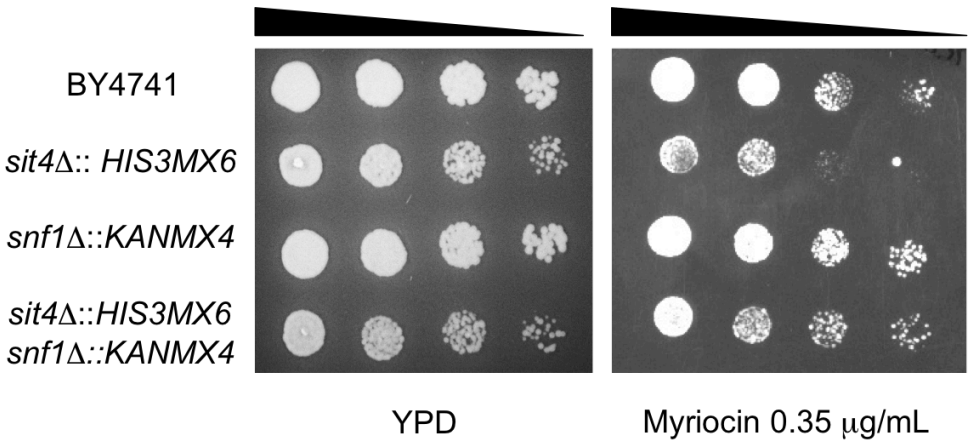


Figure 4.4. The deletion of *SNF1* suppresses myriocin sensitivity of *sit4*Δ cells. BY4741, *sit4*Δ, *snf1*Δ and *sit4*Δ*snf1*Δ cells were grown in SC medium to exponential phase, diluted to OD_{600}=0.1 and spotted in five-dilution series onto YPD plates supplemented with either ethanol 95% (vehicle) or myriocin (0.35 μg/mL). A representative image is shown.

The TORC2-dependent phosphorylation of Ypk1p upon myriocin treatment and the consequent inhibition of Orm1/2p alleviates SPT repression and enable cells to respond to sphingolipid depletion (Niles & Powers, 2012). It was previously reported that *sit4*Δ cells exhibit decreased Orm1p phosphorylation in response to

myriocin (Liu *et al.*, 2012) and our results show that this mutant strain displayed myriocin sensitivity. We therefore investigated whether the TORC2-Ypk1-Orm pathway is altered in Sit4p-deficient cells in a Snf1p-dependent manner. For this purpose, we have analyzed the TORC2-mediated phosphorylation of Ypk1p at the HM site (T662), which is responsive to alterations in sphingolipid levels (Berchtold *et al.*, 2012, Niles & Powers, 2012). The results show that the phosphorylation of T662-Ypk1p was not affected in *sit4*Δ cells but it was increased in *snf1*Δ and *sit4*Δ*snf1*Δ cells (Figure 4.5).

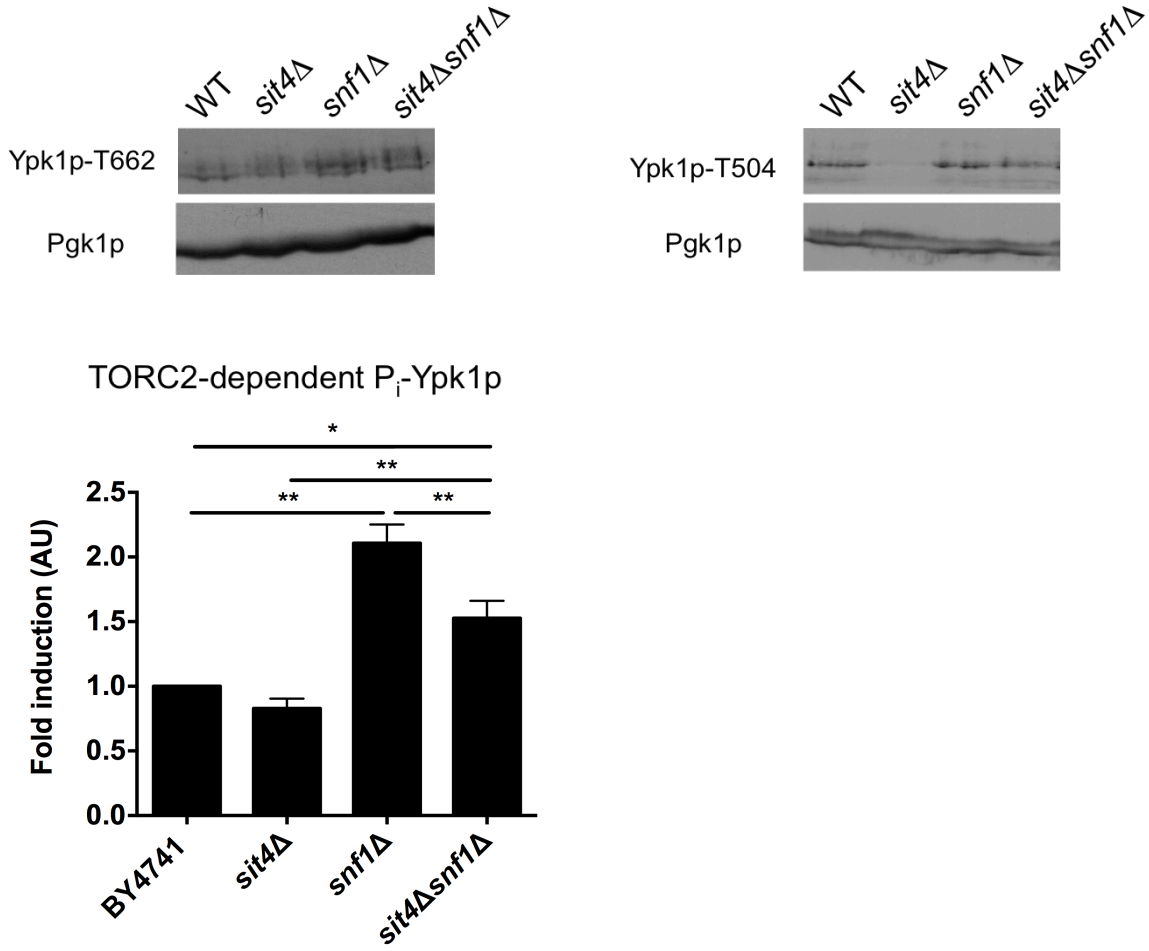


Figure 4.5. Ypk1p signalling is diminished in *sit4*Δ cells by a Snf1p-dependent mechanism. BY4741, *sit4*Δ*, snf1*Δ *and sit4*Δ*snf1*Δ cells were grown to exponential phase in YPD and the TORC2-dependent and Pkh1p-mediated phosphorylation of Ypk1p were analyzed by immunoblotting, using anti-T662-Ypk1p (top panel), anti-T504-Ypk1p (top panel), respectively, and anti-Pgk1p (loading control) as primary antibodies. A representative blot out of at least three is shown. The quantification was done by densitometry. The TORC2-dependent phosphorylation was quantified by normalization to Pgk1p levels. **p<0.01; *p<0.05.

The current model for sphingolipid regulation by TORC2 activity proposes that Pkh1p also phosphorylates Ypk1p at the A-loop and is essential for its full activation (Berchtold *et al.*, 2012, Niles & Powers, 2012). Therefore, we analyzed the activity of Pkh1p by monitoring the phosphorylation of T504 in Ypk1p (Huang *et al.*, 2012). We observed that the Pkh1p-mediated phosphorylation of Ypk1p was decreased in *sit4*Δ cells and partially restored upon deletion of *SNF1* in the mutant strain (Figure 4.5). We also took advantage a mutant allele of *YPK1*, *YPK1*^{D242A}, which is constitutively active in the absence of TORC2 function but still requires Pkh1p activity (Roelants *et al.*, 2011, Niles & Powers, 2012). In principle, the expression of this allele would not completely rescue the myriocin sensitivity of *sit4*Δ cells since it still requires functional Pkh1p. In agreement, we observed that the expression of Ypk1p^{D242A} did not suppress myriocin sensitivity of *sit4*Δ cells (Figure 4.6.A), likely due to dependence in Pkh1p-dependent phosphorylation for full activation of Ypk1p. We also observed that myriocin sensitivity could not be suppressed by increasing *PKH1* copies (Figure 4.6.B), indicating that the activation of Pkh1p rather than Pkh1p protein levels is likely a limiting factor.

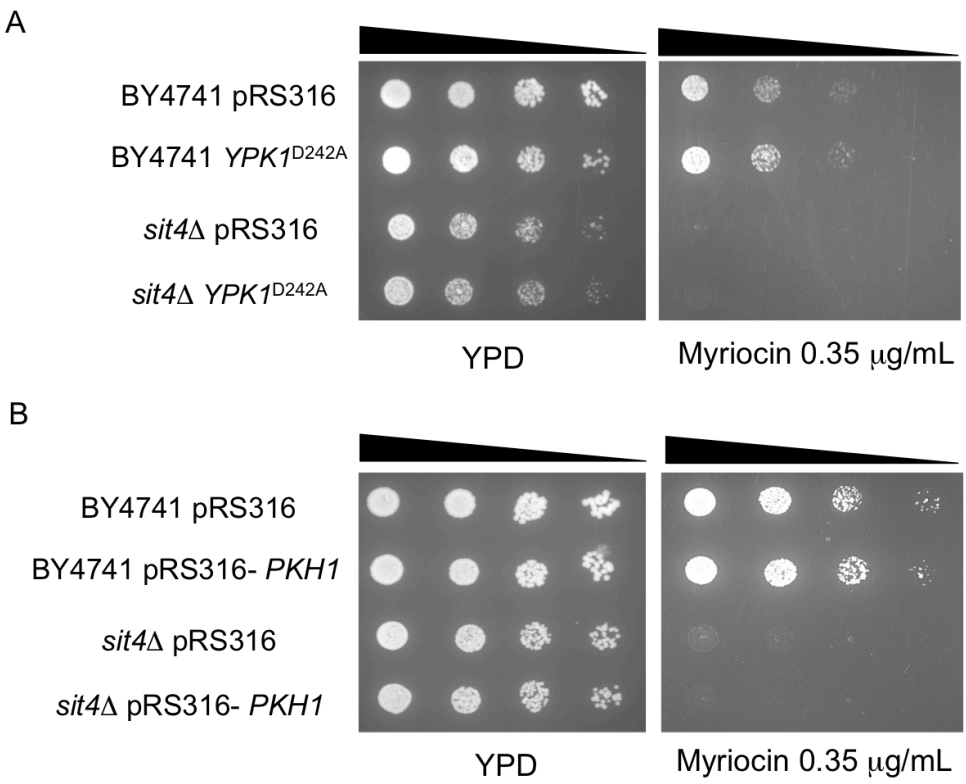


Figure 4.6. Bypassing TORC2 signalling or increased expression of *PKH1* do not suppress myriocin sensitivity of *sit4*Δ cells. BY4741 and *sit4*Δ cells carrying an

hyperactive mutant allele of *YPK1*, *YPK1*^{D242A} (A) or pRS316-*PKH1* (B) or the corresponding empty vectors were grown in SC medium to exponential phase, diluted to OD_{600}=0.1 and spotted in five-dilution series onto YPD plates supplemented with either ethanol 95% (vehicle) or myriocin (0.35 μg/mL). A representative image out of three is shown.

Taken together, our results show that TORC2-dependent phosphorylation of Ypk1p is not altered in *sit4*Δ cells but Ypk1p signalling is diminished due to decreased activity of Pkh1p. This is consistent with decreased Orm phosphorylation and myriocin sensitivity. Importantly, the deletion of *SNF1* increased the activation of TORC2-Pkh1p-Ypk1p axis in parental cells, which may explain the mild resistance of *snf1*Δ cells to myriocin. In agreement, the deletion of *YPK1* confers myriocin sensitivity to parental cells and suppressed myriocin resistance of Snf1p-deficient cells (Figure 4.7). Importantly, *SNF1* deletion partially restored the activation of this pathway in *sit4*Δ cells by increasing the TORC2 and the Pkh1p-mediated phosphorylation of Ypk1p, which correlates with enhanced TORC2-Pkh1p-Ypk1p signalling and increased resistance to myriocin displayed by *sit4*Δ*snf1*Δ cells compared to *sit4*Δ cells.

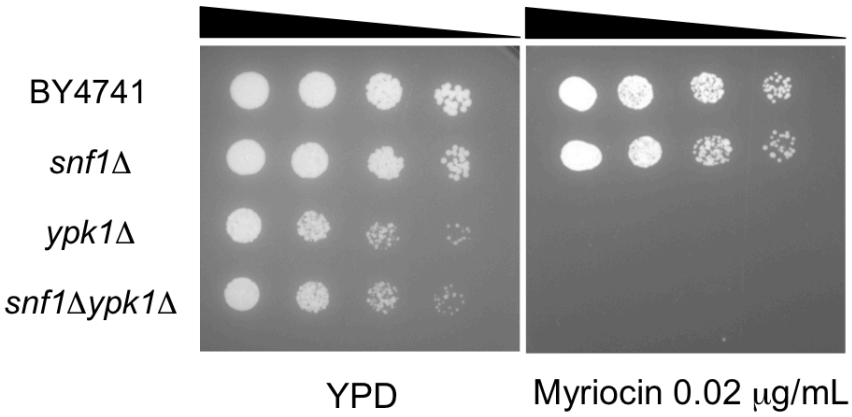


Figure 4.7. The deletion of *YPK1* confers myriocin hypersensitivity to parental and *snf1*Δ cells. BY4741, *snf1*Δ*, ypk1*Δ *and snf1*Δ*ypk1*Δ were grown in SC medium to exponential phase, diluted to OD_{600}=0.1 and spotted in five-dilution series onto YPD plates supplemented with either ethanol 95% (vehicle) or myriocin (0.02 μg/mL). A lower concentration was used due to the hypersensitivity of *ypk1*Δ cells to myriocin. A representative image out of three is shown.

4.2.4. The deletion of *SIT4* downregulates the Pkh1p-Sch9p pathway to extend lifespan in yeast

Several studies have demonstrated the important role of sphingolipids in the regulation of stress response and lifespan, in part via the activation of sphingolipid-responsive pathways, such as the Pkh1/2p-Sch9p pathway (Liu *et al.*, 2005, Huang *et al.*, 2012). The decreased Pkh1p activity in *sit4*Δ cells prompted us to monitor the phosphorylation of residue T570 in Sch9p (Huang *et al.*, 2012). Notably, both T570-Sch9p phosphorylation and Sch9p levels were decreased in *sit4*Δ cells and partially restored upon deletion of *SNF1*, suggesting that Sit4p activates the Pkh1p-Sch9p pathway in part by inhibiting Snf1p (Figure 4.8).

Since the deletion of *SIT4* (Barbosa *et al.*, 2011) and *SCH9* (Fabrizio *et al.*, 2001, Bonawitz *et al.*, 2007) also extend CLS and stress response in yeast, we aimed to investigate if the downregulation of the Pkh1p-Sch9p pathway is related to *sit4*Δ phenotypes. According to our hypothesis, the deletion of *SNF1* should be, in principle, deleterious since it partially restores the activation of the Pkh1p-Sch9p pathway by increasing Pkh1p activity and Sch9p total protein levels in *sit4*Δ cells (Figures 4.5 and 4.8).

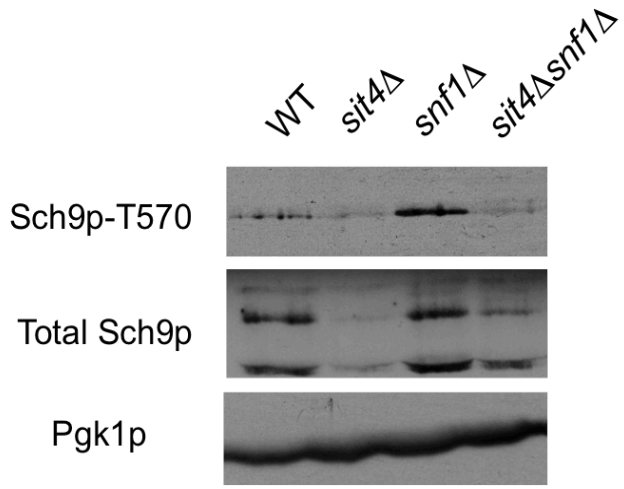


Figure 4.8. The activation of Pkh1p-Sch9p signalling pathway is decreased in *sit4*Δ cells in a Snf1p-dependent manner. BY4741, *sit4*Δ*, snf1*Δ *and sit4*Δ*snf1*Δ cells were grown to exponential phase in YPD and the Pkh1p-mediated phosphorylation of Sch9p was analyzed by immunoblotting, using anti-T570-Sch9p (top panel), anti-Sch9p (middle panel) and anti-Pgk1p (loading control) as primary antibodies. A representative blot out of at least three is shown.

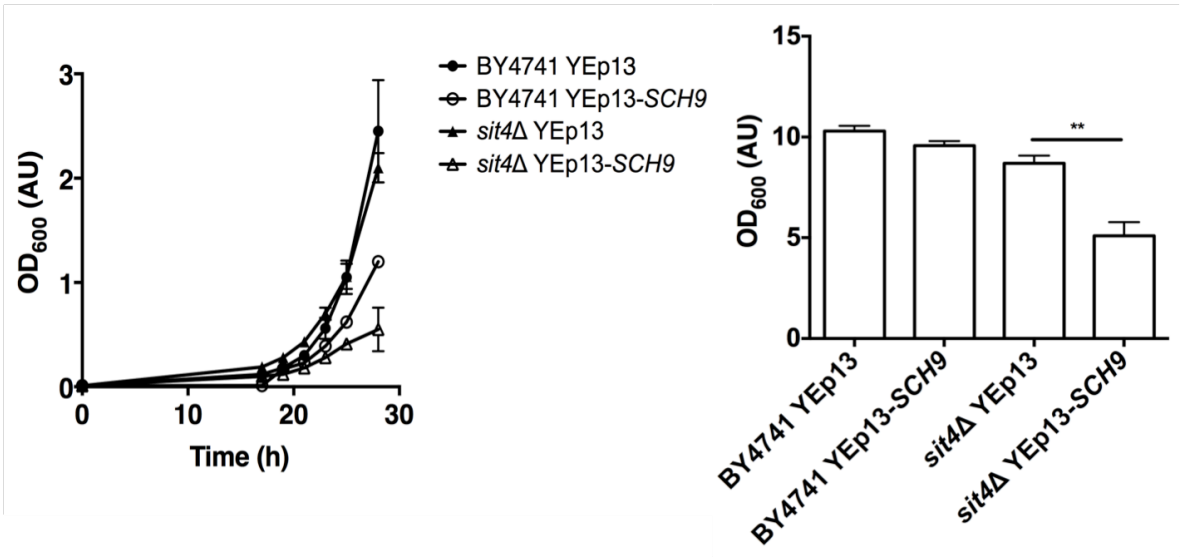
In agreement, the deletion of *SNF1* in *sit4*Δ cells leads to decreased cell growth, hydrogen peroxide sensitivity, abolishes CLS extension and promotes impaired mitochondrial function (Pereira *et al*., *unpublished results*). To provide further evidence that the restoration of Sch9p levels contribute to these phenotypes in *sit4*Δ*snf1*Δ cells, *SCH9* was overexpressed in *sit4*Δ cells. In fact, the overexpression of *SCH9* impaired cell growth, particularly in the stationary phase (Figure 4.9.A), abolished hydrogen peroxide resistance (Figure 4.9.B) and reduced oxygen consumption (Figure 4.9.C) and glycogen storage (Figure 4.9.D) in *sit4*Δ cells. Importantly, we also observed that the overexpression of *SCH9* partially shortened the CLS extension of Sit4p-deficient cells (Figure 4.9.E).

4.3. Discussion

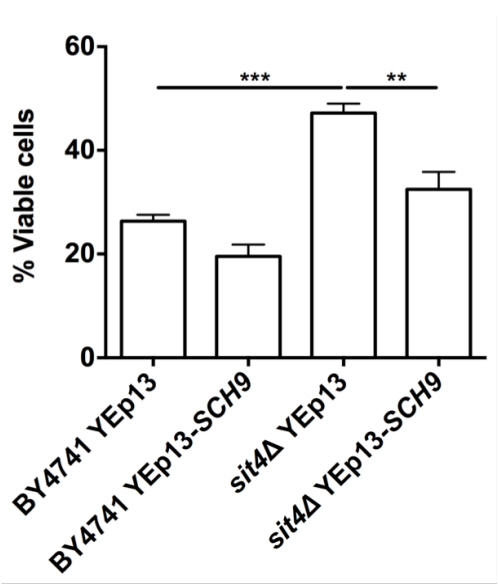
The role of sphingolipids in aging and age-associated pathologies, such as neurodegenerative diseases and cancer, has been extensively reported. In fact, alterations on sphingolipids metabolism deregulate cell growth and survival and general stress responses with impact on the onset of these diseases (Kolter, 2011). However, the molecular mechanisms by which signalling pathways are activated in response to alterations in sphingolipid biosynthesis or turnover are not yet fully understood. This clarification is pivotal to understand sphingolipid functions in more detail and define new strategies to improve human health and extend lifespan.

In this study, we report that Sit4p, the yeast orthologue of human phosphatase PP6, is not only an effector but also a regulator of sphingolipids metabolism. Our results show that Sit4p is required for proper regulation of sphingolipid metabolism, which is consistent with Sit4p-deficient cells presenting higher LCBPs/LCBs ratio and reduced ceramide content during exponential growth (Vilaça, 2014). Regarding the sphingolipids biosynthetic pathway, we provide evidence that Sit4p takes part of a novel mechanism in which this phosphatase plays a key role in the regulation of the *YPC1* and *LAG1* expression, since *YPC1* expression was decreased and *LAG1* expression was increased in *sit4*Δ cells.

A


BY4741 YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13
sit4Δ YEp13-SCH9
OD600 (AU)
Time (h)
0
10
20
30
1
2
3
5
15
**
BY4741 YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13
sit4Δ YEp13-SCH9

B


% Viable cells
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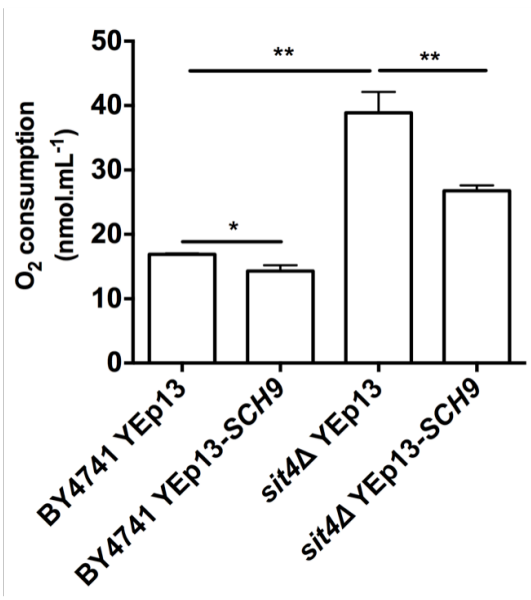
**
BY4741 YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13
sit4Δ YEp13-SCH9

C


O2 consumption (nmol.mL-1)
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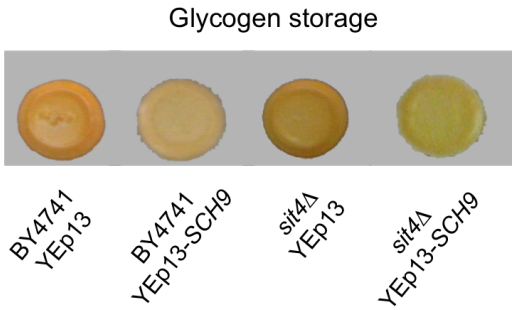
BY4741 YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13
sit4Δ YEp13-SCH9

D


Glycogen storage
BY4741 YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13
sit4Δ YEp13-SCH9

E

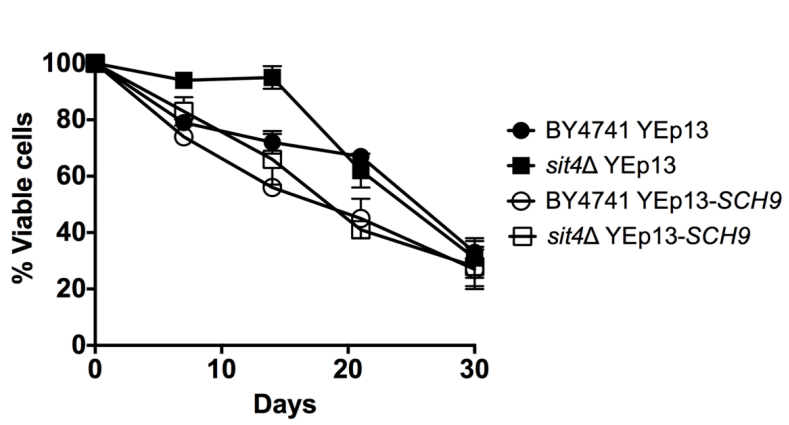

% Viable cells
Days
BY4741 YEp13
sit4Δ YEp13
BY4741 YEp13-SCH9
sit4Δ YEp13-SCH9

Figure 4.9. The overexpression of *SCH9* abolishes hydrogen peroxide resistance, increased oxygen consumption and the glycogen storage phenotype of *sit4*Δ cells. **A.** Growth curves of BY4741 and *sit4*Δ cells carrying the empty vector (YEp13) or overexpressing *SCH9* (YEp13-*SCH9*) grown in SC-glucose to early exponential phase and maintained overtime. The OD_{600} values read after 40 hours of incubation is depicted on the right. Values are mean ± SD of three independent experiments. **p<0.01 **B.** BY4741 and *sit4*Δ cells carrying the empty vector (YEp13) or overexpressing *SCH9* (YEp13-*SCH9*) were grown in SC-medium to the exponential phase and exposed to 1.5 mM H_2O_2 for 60 min. Cell viability was expressed as the percentage of the colony-forming units (treated cells versus untreated cells). Values are mean ± SD of at least three independent experiments. ***p<0.001; **p<0.01. **C.** Oxygen consumption rate was measured in cells grown to PDS phase. Values are mean ± SD of at least three independent experiments. **p<0.01; *p<0.05. **D**. Analysis of glycogen content in BY4741 and *sit4*Δ cells carrying the empty vector (YEp13) or overexpressing *SCH9* (YEp13-*SCH9*). Cells were grown in SC-medium to exponential phase, diluted to OD_{600}=0.1 and spotted onto YPD agar. After 3 days of growth, iodine staining was performed by exposing cells to iodine vapor. A representative image out of three is shown. **E.** Analysis of chronological lifespan in BY4741 and *sit4*Δ cells carrying the empty vector (YEp13) or overexpressing *SCH9* (YEp13-*SCH9*). Values are mean ± SD of at least three independent experiments.

The downregulation of the phytoceramidase *YPC1* gene may account for the specific accumulation of phytoceramides observed in *sit4*Δ cells, which then becomes the substrate for the synthesis of α-hydroxylated phytoceramides (Vilaça, 2014). Importantly, the induction of the ceramide synthase *LAG1* gene may occur either as a feedback loop mechanism in response to reduced ceramide levels observed in *sit4*Δ cells. A reduction in ceramide synthase activity predicts an increase in LCBs, as observed in *avo3-30* (Aronova *et al.*, 2008) and *sit4*Δ cells (Vilaça, 2014). More recently, Ypk1p was shown to activate Lac1p and Lag1p by phosphorylation at Ser23 and Ser24 (catalytic domain), which is antagonized by calcineurin (Muir *et al.*, 2014). It would be interesting to assess ceramide synthase activity, and the Ypk1p-mediated phosphorylation of the catalytic domain of ceramide synthase to establish a connection between the activity of ceramide synthase and a reduction in ceramide content observed in Sit4p-deficient cells (Vilaça, 2014). It is noteworthy that calcineurin appears to act in parallel to these

pathways since FK506-induced inhibition of calcineurin further enhances the myriocin sensitivity of *sit4*Δ cells (Figure 4.S1).

In cells devoid of Sit4p, sphingolipid flux is preferentially shunted to favor the accumulation of phytoceramides and drive the synthesis of α-hydroxylated phytoceramide species (Vilaça, 2014). Importantly, the levels of α-hydroxylated phytoceramides were previously associated with proper mitochondrial function in yeast (Kitagaki *et al.*, 2007). Thus, the observed increase in the content of α-HO phytoceramide species may account for improved mitochondrial function displayed by *sit4*Δ cells already at log phase (Jablonka *et al.*, 2006, Barbosa *et al.*, 2011, Vilaça, 2014).

It was previously reported that the phosphorylation of Orm1p but not Orm2p was decreased in *sit4*Δ cells in response to both rapamycin and myriocin (Liu *et al.*, 2012). According to the model proposed by Shimobayashi *et al.,* (Shimobayashi *et al.*, 2013), the downregulation of TORC1 relieves Sit4p inhibition and therefore Npr1p kinase becomes activated to phosphorylate and activate Orm1p, thus stimulating complex sphingolipid biosynthesis. Here we demonstrate that decreased Orm phosphorylation in *sit4*Δ cells may also be attributed to decreased Ypk1p signalling. Although the phosphorylation of Ypk1p by TORC2 was unaltered in Sit4p-deficient cells, full activation of Ypk1p still requires its phosphorylation by Pkh1p. In this study, we provide evidence that overall activation of Ypk1p is decreased in *sit4*Δ cells. In agreement, we showed that Pkh1p-driven phosphorylation of Ypk1p at T504 is decreased in *sit4*Δ cells. It was previously reported that the phosphorylation of Ypk1p by Pkh1p at T504 is also required for efficient endocytosis (deHart *et al.*, 2002). In this scenario, reduced phosphorylation of Ypk1p-T504 may also explain defective endocytosis previously reported for *sit4*Δ cells (Burston *et al.*, 2009).

We also show that a hyperactive form of Ypk1p, which bypasses TORC2 signalling, was unable to suppress myriocin sensitivity because it likely requires the Pkh1p-dependent phosphorylation for full activation of Ypk1p. Furthermore, decreased Ypk1p activation in *sit4*Δ cells correlates well with reduced Orm phosphorylation observed in the mutant strain at basal conditions and in response to myriocin (Liu *et al.*, 2012). Consistently, *ypk1*Δ cells are more sensitive to myriocin (Roelants *et al.*, 2004, Tanoue *et al.*, 2005), a feature that is shared with

Sit4p-deficient cells. In addition, *sit4*Δ cells are more sensitive to heat stress (Ruiz-Roig *et al.*, 2010) (and our observations, data not shown), which promotes the *de novo* sphingolipid biosynthesis, and Ypk1p-Orm signalling is required for heat stress adaptation (Sun *et al.*, 2012). Overall, decreased Ypk1p-dependent inhibition of Orm1/2p in *sit4*Δ cells favors SPT inhibition, likely contributing to downregulate sphingolipid biosynthesis. In agreement, ceramide production is decreased in this mutant (Vilaça, 2014).

Altogether, we propose that Sit4p is a master regulator of sphingolipid biosynthesis and metabolism by integrating signalling derived from TORC1 and TORC2 branches. TORC1 inhibits Sit4p, which in turn decreases Npr1p activity. As a result, Orm1/2p phosphorylation decreases and they become less active, thus reducing the flux of ceramide and complex sphingolipid synthesis in the Golgi apparatus (Shimobayashi *et al.*, 2013). Our study provides evidence that deletion of *SIT4* also decreases *de novo* sphingolipids biosynthesis pathway by decreasing Pkh1p-Ypk1p signalling (by Snf1p-dependent mechanisms), which reduces Orm phosphorylation. Therefore, SPT is inhibited and the biosynthesis of ceramide is diminished (Vilaça, 2014). Interestingly, it was shown that Sit4p co-purifies with Tor2p (Yan *et al.*, 2006), which further supports a regulatory role of Sit4p in TOR readouts. Additional studies are required to demonstrate if Sit4p physically interacts with Tor2p to specifically regulate TORC2 functions, since Tor2p can also be present on the TORC1 protein complex (Loewith & Hall, 2011).

Mechanistically, we demonstrate that Snf1p is required for the regulation of these molecular events. In fact, we provide evidence that the deletion of *SNF1* increases the TORC2-Pkh1p-Ypk1p signalling in parental cells, likely contributing to mild myriocin resistance. In agreement, the deletion of *YPK1* conferred myriocin hypersensitivity to Snf1p-deficient cells, further demonstrating that Snf1p is epistatically upstream of Ypk1p. Notably, the pathway is also upregulated in *snf1*Δ*sit4*Δ cells since TORC2-Pkh1p-Ypk1p signalling is increased compared to *sit4*Δ cells. As a result, *snf1*Δ*sit4*Δ cells became more resistant to sphingolipid depletion induced by myriocin in relation to *sit4*Δ cells.

Huang *et al.* demonstrated that sphingolipids regulate CLS by modulating the activation of the Pkh1/2p-Sch9p pathway, which responds to LCBs (Huang *et al.*, 2012). In this study, we demonstrated that Sit4p controls the Pkh1p-Sch9p pathway by regulating Pkh1p activity and Sch9p protein levels, the latter either by

expression or turnover. Importantly, the deletion of *SIT4* promotes CLS extension and improves stress response and mitochondrial function in yeast (Barbosa *et al.*, 2011). We propose that the downregulation of the Pkh1p-Sch9p pathway also contribute to these phenotypes in *sit4*Δ cells. This is supported by the fact that the activation of the pathway promoted by the deletion of *SNF1*, which increases Pkh1p activity and partially restores Sch9p levels, abolished oxidative stress resistance, mitochondrial function and the extended CLS exhibited by *sit4*Δ cells (Pereira *et al*., *unpublished results*). Notably, Snf1p physically interacts with Sin4p (Kuchin *et al.*, 2000), a subunit of the RNA polymerase II mediator complex that may regulate *SCH9* expression. Further studies are necessary to test this hypothesis.

Nevertheless, we may not exclude possible effects related to Snf1p functions, which is essential for the metabolic adaptation upon glucose starvation and induction of respiration (Young *et al.*, 2003, Hardie, 2007). Moreover, the overexpression of *SCH9* in *sit4*Δ cells produced similar effects as observed for *sit4*Δ*snf1*Δ double mutants. We thus conclude that Sit4p modulates the activation of the Pkh1/2p-Sch9p pathway by a Snf1p-dependent mechanism to regulate stress response and lifespan in yeast.

Sit4p-deficient cells accumulate LCBPs and present decreased ceramide levels during exponential phase (Vilaça, 2014), which corresponds to the sphingolipid profile exhibited by stationary phase-grown cells (Lester *et al.*, 2013). This may indicate that Sit4p deficiency may reprogram sphingolipid metabolism already during growth to precondition cells to better survive in the stationary phase and extend CLS. Although the link between the accumulation of LCBPs and lifespan is not yet fully understood (Lester *et al.*, 2013), a recent study demonstrated that knocking out *LCB4* abolished the anti-aging effect of CR (Tang *et al.*, 2008), which indicates that the formation of LCBPs is important for lifespan extension. Apart from it, *sit4*Δ cells present several characteristics of stationary phase-grown cells already during exponential growth, namely derepression of mitochondrial function (Jablonka *et al.*, 2006), enhanced oxidative stress resistance (Barbosa *et al.*, 2011) and glycogen storage (Jablonka *et al.*, 2006), which has been associated with longevity in yeast. As a result, sphingolipid metabolism is redirected and overall cell fitness is improved upon Sit4p deficiency already during growth, contributing to extend CLS in yeast. Our results support a

model in which Sit4p is activated by ceramide but also regulates sphingolipids metabolism by integrating molecular events involving the TORC2-Ypk1p signalling and Orm phosphorylation to modulate the sphingolipid biosynthetic pathway.

4.4. Experimental Procedures

4.4.1. Yeast strains and growth conditions

S. cerevisiae BY4741 was the parental strain of all haploid derivatives used in this study (Table 4.5.1). Yeast cells were grown aerobically at 26°C in a gyratory shaker (at 140 r.p.m.), with a ratio of flask volume/ medium volume of 5:1. The growth media used were YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose] and synthetic complete (SC) drop-out medium containing 2% (w/v) glucose, 0.67% yeast nitrogen base without amino acids and supplemented with appropriate amino acids (80 mg histidine L^{-1}, 400 mg leucine L^{-1}, 80 mg tryptophan L^{-1} and 80 mg uracil L^{-1}). Gene disruption was done by conventional methods and the proper integration of the cassettes was confirmed by PCR.

4.4.2. Stress resistance and chronological lifespan

For H_2O_2 resistance assay, cells were grown to the exponential phase (OD_{600} = 0.6) and exposed to 1.5 mM H_2O_2 (Merck) for 60 min. The CLS assay was performed as described (Fabrizio & Longo, 2003). Briefly, overnight cultures were diluted to OD_{600} = 0.6, grown for 72 h (to stationary phase; considered T_0 in the CLS assay) and kept in medium at 26°C. For both assays, cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % (w/v) agar and expressed as the percentage of the colony-forming units after growth at 26ºC for 3 days (time *T* vs. T_0 (when viability was considered 100%) for the CLS assay; treated vs. untreated cells for H_2O_2 resistance). Values are mean ± SD of at least three independent experiments. To assess cellular resistance to myriocin (Sigma-Aldrich) or phytosphingosine (Sigma-Aldrich), yeast cells were grown in SC-medium to exponential phase, diluted to OD_{600}=0.1, spotted onto

YPD plates containing these compounds at the indicated concentrations and incubated at 26ºC for 2-3 days.

4.4.3. Sphingolipid profiling

Yeast cells were grown in SC-glucose medium and 1.9 × 10^9 cells were collected at exponential phase. Cell pellets were ressuspended in 1 mL of lipid extraction solvent: 50% (v/v) iso-propanol, 10% (v/v) diethyl ether, 2% (v/v) pyridine, 25% (v/v) ammonia. A 200 µL volume of glass beads were added into a screw cup 2 mL plastic tubes. Tubes were shaken in a bead better five times, 3 min on, 1 min off at 4°C. The content of the tubes was poured into a 13 × 100 mm glass tubes. An additional 1 mL solvent was used to wash the plastic tubes and was added into the glass tubes. The tubes containing 2 mL solvent with cells and glass beads were dried in an analytical nitrogen evaporator (N-EVAP). The dried samples were sent to the Lipidomic Core at the Medical University of South Carolina for lipid analysis. Levels of ceramide, long-chain sphingoid bases and their phosphorylated forms were measured by the high-performance liquid chromatography/mass spectrometry (LC-MS/MS) methodology as previously described (Bielawski *et al.*, 2010). Analytical results of lipids were expressed as pmol sphingolipid/total cell number.

4.4.4. Enzymatic activities and oxygen consumption

The activity of β-galactosidase was determined as previously described (Teixeira *et al.*, 2014). Oxygen consumption rate was measured for 3 x 10^8 cells in PBS buffer (pH 7.4), using an oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software.

4.4.5. Western Blot analysis

To evaluate the TORC2-dependent phosphorylation (T662) and the Pkh1p-mediated phosphorylation (T504) of Ypk1p, the Pkh1p-mediated activation of Sch9p (T570) and total Sch9p protein levels, cells were harvested and subjected to alkaline lysis. Proteins (50 µg) were resolved by SDS–PAGE using 8.5%

polyacrylamide gels and blotted onto a nitrocellulose membrane (GE Healthcare). The primary antibodies used were mouse anti-phospho-T662-Ypk1p (kindly provided by Dr. Robbie Loewith, 1:500), rabbit anti-phospho-T504 Ypk1p (Phospho-PKC (pan) zeta Thr410 (190D10)); Cell Signalling Technology, 1:1,000), rabbit anti-phospho-T570-Sch9p (kindly provided by Dr. Robbie Loewith, 1:10,000), rabbit anti-Sch9p (kindly provided by Dr. Robert Dickson, 1:1,000) and mouse anti-Pgk1p (Invitrogen, 1:30,000). Subsequently, the membrane was incubated with the secondary antibody anti-mouse IgG-peroxidase (Molecular Probes) or anti-rabbit (Sigma) at a 1:5,000 dilution. Immunodetection was done using the Lumigen HRP chemiluminescent substrate (GE Healthcare).

4.4.6. Glycogen content

Yeast cells were grown in SC-medium to exponential phase, diluted to OD_{600}=0.1, spotted onto YPD plates and incubated for 3 additional days. The iodine staining was performed by exposing cells to iodine vapor (Sigma-Aldrich) (Wilson *et al.*, 2002).

4.4.7. Statistical analysis

Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's t-test. The 0.05 probability level was chosen as the point of statistical significance throughout. Statistical analyses were carried out using GraphPad Prism Software v5.01 (GraphPad Software).

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4.5. Supplementary Information

Table 4.5.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4741	Mata *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* [YEp357-*YPC1*, YEp357-*YDC1*, YEp357-*LAG1,* YEp357-*LAC1,* p416MET25-*YPK1*^{D242A}, pRS316, pRS316-*PKH1,* YEp13,YEp13-*SCH9*]	EUROSCARF This study
*sit4*Δ::KanMX4	BY4741 *sit4*Δ::*KanMX4* [p416MET25-*YPK1*^{D242A}, pRS316, pRS316-*PKH1,* YEp13,YEp13-*SCH9*]	EUROSCARF This study
*sit4*Δ::HIS3MX6	BY4741 *sit4*Δ::*HIS3MX6* [YEp357-*YPC1*, YEp357-*YDC1*, YEp357-*LAG1,* YEp357-*LAC1*]	Vilaça, 2014 This study
*snf1*Δ	BY4741 *snf1*Δ::*KanMX4*	EUROSCARF
*sit4*Δ*snf1*Δ	BY4741 *sit4*Δ::*HIS3MX6 snf1*Δ::*KanMX4*	Pereira *et al.*
*lcb4*Δ	BY4741 *lcb4*Δ::*KanMX4*	EUROSCARF
*sit4*Δ*lcb4*Δ	BY4741 *sit4*Δ::*HIS3MX6 lcb4*Δ::*KanMX4*	This study
*ypk1*Δ	BY4741 *ypk1*Δ::*KanMX4*	EUROSCARF
*snf1*Δ*ypk1*Δ	BY4741 *snf1*Δ::*LEU2 ypk1*Δ::*KanMX4*	This study

Harboring plasmids are shown in square brackets.

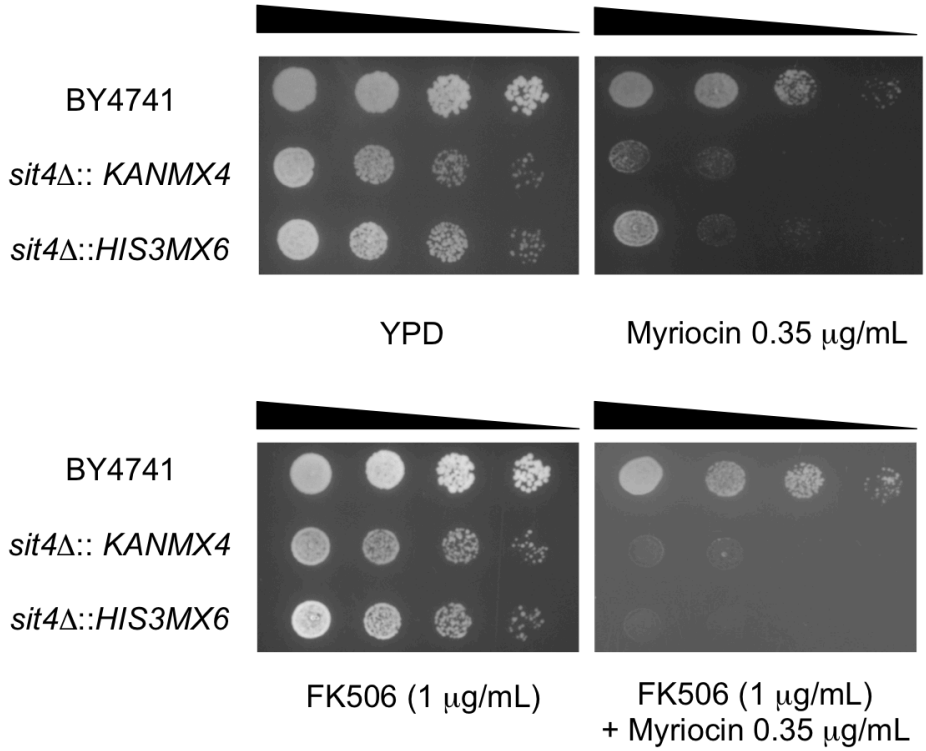

Figure 4.S1. Calcineurin inhibition further increases the myriocin sensitivity of Sit4p-deficient cells. BY4741 and *sit4*Δ cells with different selection markers were grown in SC medium to exponential phase, diluted to OD_{600}=0.1 and spotted in five-dilution series onto YPD agar supplemented with either ethanol 95% (vehicle) or myriocin (0.35 μg/mL) and calcineurin inhibitor FK506 (1 μg/mL) as indicated. A representative image out of three is shown.

CHAPTER V

General discussion and future perspectives

5.1. General discussion and future perspectives

Sphingolipids have come to the foreground of research area in the past few years due to the recognition of their importance as bioactive molecules involved in the regulation of overall cellular metabolism and fate (Cowart *et al.*, 2006, Hannun & Obeid, 2008, Liu *et al.*, 2013, Rego *et al.*, 2014). Their importance is reinforced by the fact that sphingolipid metabolism is highly conserved among eukaryotes, ranging from yeast to mammals (Cowart *et al.*, 2006, Hannun & Obeid, 2008, Liu *et al.*, 2013, Rego *et al.*, 2014). Most importantly, subtle variations in the "sphingolipid rheostat" have a significant impact in cell physiology and fate (Cowart *et al.*, 2006, Hannun & Obeid, 2008, Liu *et al.*, 2013, Rego *et al.*, 2014). As a result, sphingolipid metabolism is tightly regulated. In addition, sphingolipids actively modulate important cellular processes, such as cell cycle arrest, apoptosis, differentiation, senescence and stress responses, and also contribute to the etiology of several human disorders (Dickson, 2008, Hannun & Obeid, 2008, Young *et al.*, 2013).

In *S. cerevisiae*, the homologue of mammalian neutral sphingomyelinase type 2, Isc1p, is required for proper oxidative stress resistance and chronological lifespan. In fact, *isc1*Δ cells exhibit severe mitochondrial dysfunction, hydrogen peroxide hypersensitivity and premature aging, which is, in part, associated with increased intracellular levels of iron and Yca1p-mediated cell death by apoptosis (Kitagaki *et al.*, 2007, Almeida *et al.*, 2008, Kitagaki *et al.*, 2009). The activation of the ceramide-activated protein phosphatase Sit4p and the Hog1p protein kinase in response to ceramide signalling, are associated with these phenotypes, as the deletion of *SIT4* (Barbosa *et al.*, 2011) and *HOG1* (Barbosa *et al.*, 2012) suppresses *isc1*Δ associated phenotypical traits.

The role of the sphingomyelinase Isc1p in signalling transduction involved in stress response and chronological aging is yet not fully understood. The knowledge of the involvement of Isc1p-driven ceramide signalling in the regulation of fundamental biological processes, such as autophagy and related mechanisms, is also still scarce.

This thesis aimed to explore the involvement of the nutrient-sensing Target Of Rapamycin Complex 1 (TORC1) and its downstream effector, the AGC protein

kinase Sch9p, in *isc1*Δ phenotypes. This work demonstrates that the TORC1-Sch9p pathway is activated in cells lacking Isc1p and contributes to *isc1*Δ phenotypes since the deletion of *TOR1* or *SCH9* suppresses the premature aging, oxidative stress sensitivity and mitochondrial dysfunctions displayed by *isc1*Δ cells. Importantly, this is correlated with autophagic defects exhibited by the mutant strain.

More work is required to characterize the molecular mechanism(s) by which Isc1p regulates the activation of the TORC1-Sch9p pathway. TORC1 activity is highly sensitive to nutrient availability but also integrates signals from various stresses and growth factors. In yeast and mammals, TORC1 is activated by aminoacid availability. Withdrawal of branched-chain amino acids, in particular leucine, leads to a strong reduction of TORC1 activity, implying that leucine is the most potent activator of TORC1 (Binda *et al.*, 2009). In yeast, TORC1 activity can also be stimulated by addition of other nitrogen sources, such as ammonium ions, albeit with different efficiencies. Interestingly, depletion of intracellular glutamine levels mimics inactivation of TORC1, suggesting a direct role for glutamine in the activation of TORC1 (Crespo *et al.*, 2002, Binda *et al.*, 2009). In yeast and mammals, various direct activators of TORC1 have also been identified, namely the small GTPase Rheb, which is required for the activation of TORC1 in response to growth factors and possibly by aminoacids (Sancak *et al.*, 2008). In addition, some studies have also shown that sphingolipids, especially ceramide, alters nutrient sensing and internalization (Guenther *et al.*, 2008). Since TORC1 is responsive to aminoacids, the deregulation of nutrient sensing may affect the activation of the TORC1-Sch9p pathway in *isc1*Δ cells.

Interestingly, inhibition of translation with cycloheximide causes a pronounced increase in TORC1 activity presumably by triggering an increase in the concentration of free aminoacids in the cytoplasm (Urban *et al.*, 2007). Interestingly, reduced ribosome biogenesis results in a dramatic increase in TORC1 activity (Lempiainen *et al*., 2009). In Isc1p-deficient cells, it was observed reduced expression of genes encoding for ribosomal proteins and corresponding subunits (Almeida *et al.*, 2008). Therefore, it is possible that blocking ribosome biogenesis, like translation and protein synthesis inhibition, may lead to an increase in free aminoacids that subsequently activates TORC1 in *isc1*Δ cells. This hypothesis needs further clarification.

Apart from TORC1, Sch9p is also responsive to LCBs and this study shows that it also senses ceramide signalling to regulate Hog1p. As a result, Sch9p activity is responsive to nutrient-derived TORC1 and Isc1p-driven ceramide signalling inputs to regulate mitochondrial function, oxidative stress sensitivity and lifespan in yeast. Overall, our data support a model in which Isc1p regulates mitochondrial function and chronological lifespan in yeast through the TORC1-Sch9p pathway, which acts as a central axis to integrate upstream Isc1p-driven ceramide signalling signals to downstream effectors Sch9p and Sit4p.

Although the role of Snf1p, the yeast homologue of AMPK, as a downstream target of TORC1 is still controversial (Zhang *et al.*, 2011), the hyperactivation of the TORC1 could then lead to the downregulation of Snf1p. The assumption of decreased activation of Snf1p could also explain phenotypic similarities between strains lacking Isc1p and Snf1p, namely the inability to grow on non-fermentable carbon sources, decreased ion tolerance, aberrant cell wall characteristics and increased ROS accumulation (Kuchin *et al.*, 1993, Alepuz *et al.*, 1997, Tabuchi *et al.*, 2006, Weinberger *et al.*, 2010, Barbosa *et al.*, 2012). In addition, downregulation of Snf1p could also account for autophagic defects, as Snf1p is a positive regulator of autophagy (Jewell *et al.*, 2013). These hypothesis need to be clarified in more detail.

Macroautophagy plays an important regulatory role in mitochondrial function, stress response and aging (Seo *et al.*, 2010). Since these processes are compromised in Isc1p-deficient cells, this thesis also aimed to evaluate the impact of Isc1p-driven ceramide signalling on macroautophagy and crucial mitochondrial quality control mechanisms, namely mitochondrial dynamics and mitophagy. Reduced autophagic flux displayed by *isc1*Δ cells is attributed to primary defects in vesicular trafficking and vacuolar proteolysis and morphology and ability to maintain the proton motive force in the vacuole. These phenotypes were abolished by downregulation of TORC1 and its downstream effectors, Sch9p and Sit4p, which integrate nutrient and stress signals from TORC1 with ceramide signalling from Isc1p. As a result, it is conceivable to assume that these proteins kinases and the Sit4p protein phosphatase regulate the phosphorylation status or the catalytic activity of proteins involved in vesicular trafficking, such as Rab GTPases, tethering factors, and SNAREs protein machinery (Cai *et al.*, 2007). In addition, alterations in sphingolipid profiling previously reported (Barbosa *et al.*, 2011) could

also impair vesicular trafficking in *isc1*Δ cells. In this case, it would be important to determine which sphingolipid species could possibly play a key role in this process.

On the other hand, we show that the deletion of *ISC1* leads to the hyperactivation of mitophagy, presumably as an adaptive response to mitochondrial dysfunction since this was correlated with loss of cell viability of *isc1*Δ cells. Importantly, the deletion of *TOR1*, *SCH9*, *SIT4* and *HOG1* attenuated the induction of mitophagy in respiratory conditions and this was correlated with the suppression of mitochondrial fragmentation and improvement of mitochondrial function. This could possibly indicate that, when Isc1p is translocated to the mitochondria upon the transition to respiratory conditions (PDS phase), the regulation of sphingolipid metabolism by Isc1p at this organelle may be important to modulate ceramide pools and control the activation of the TORC1-Sch9p, Sit4p and Hog1p signalling pathways, contributing to maintain proper mitochondrial function and dynamics and oxidative stress resistance. As a result, cells are better conditioned to endure the stationary phase.

Isc1p-deficient cells also displayed higher levels of mitochondrial fission protein Dnm1p associated with unbalanced mitochondrial fission, contributing to mitochondrial fragmentation, oxidative stress sensitivity and shortened lifespan of *isc1*Δ cells. How Isc1p regulates Dnm1p protein levels (which are increased in *isc1*Δ cells) and activity remains to be understood. According to our hypothesis, in respiratory conditions, mitochondrial fragmentation leads to the hyperactivation of mitophagy, presumably in response to mitochondrial dysfunction. Importantly, mitochondrial dysfunction is associated with apoptotic cell death in *isc1*Δ cells. Since Dnm1p is also involved in apoptosis (Frank *et al.*, 2001), the recruitment of Dnm1p and impairment of mitochondrial dynamics may constitute one of the primary events leading to apoptosis in *isc1*Δ cells. Supporting a regulatory role in mitochondrial dynamics, we show that Isc1p and Dnm1p physically interacted *in vitro*. It is possible that alterations in ceramide content (dependent of Isc1p activity) leading to dysfunction of mitochondria may constitute a signal that promotes the recruitment of Dnm1p, leading to the segregation and degradation of damaged mitochondria by mitophagy as an adapative response to cope with mitochondrial dysfunction. Further studies are needed to test this hypothesis.

Our lab was able to show that the deletion of *SIT4* extends lifespan in yeast by boosting mitochondrial function (mitochondrial catabolic derepression) and stress response (Barbosa *et al.*, 2011). Although Sit4p functions downstream of Isc1p (Barbosa *et al.*, 2011), the phosphatase is also downregulated by TORC1 (Di Como & Arndt, 1996, Jacinto *et al.*, 2001, de Virgilio & Loewith, 2006). In addition, Sit4p was recently linked to sphingolipid metabolism, as TORC1 controls the turnover of complex sphingolipids in a Sit4p-dependent manner (Shimobayashi *et al.*, 2013). However, how TORC1 effector Sit4p integrates nutrient and sphingolipid signalling to regulate lifespan is not yet established. On this basis, the work aimed to establish a functional association between Sit4p and sphingolipid metabolism and evaluate the impact on cell survival. This work demonstrates that Sit4p regulates the sphingolipid biosynthetic pathway by a transcriptional regulatory mechanism involving the ceramide synthase *LAG1* and the ceramidase *YPC1*. Importantly, increased LCBPs/LCBs ratio and the preferential shunting of sphingolipid metabolism towards the synthesis of α-hydroxylated phytoceramide species (Vilaça, 2014), which have been implicated on proper mitochondrial function (Kitagaki *et al.*, 2007), are possible traits associated with CLS extension imparted by *SIT4* deletion. Overall ceramide production is decreased in *sit4*Δ cells (Vilaça, 2014) and this was correlated with reduced activation of the Pkh1p-Ypk1p pathway and decreased Orm phosphorylation by a Snf1p-dependent mechanism. Moreover, the Pkh1/2p-Sch9p pathway is significantly downregulated in this mutant and Snf1p also mediates this effect. How Snf1p, which is involved in glucose sensing (Hardie, 2007), regulates sphingolipid metabolism needs to be clarified in more detail at a molecular level. At the cellular level, it has been demonstrated that ceramides impair insulin signalling and intracellular handling of glucose and lipids with resulting deleterious effects on cellular metabolism (Larsen & Tennagels, 2014). Therefore, Snf1p may constitute an additional level of regulation to couple sphingolipid signalling with glucose metabolism, which is particularly relevant in a therapeutic context (chronic hyperglycemia or hyperlipidemia or inflammatory diseases) (Larsen & Tennagels, 2014). Therefore, further studies could be useful to discriminate possible regulatory roles of Snf1p in sphingolipid and glucose metabolism and how they are coupled at a cellular level.

Importantly, the downregulation of the Pkh1/2p-Sch9p pathway was associated with improved mitochondrial fitness and extended CLS upon Sit4p

deficiency. Since Sit4p integrates TORC1-associated nutrient signalling with Isc1p-driven ceramide signalling and also regulates the activation of the Pkh1/2p-Sch9p pathway, our results provide evidence that Sit4p is not only an effector but also a key regulator of sphingolipid metabolism and is involved in a regulatory network of interacting pathways that integrate signals from TORC1-mediated nutrient sensing with sphingolipid metabolism to regulate cell growth and longevity.

The deletion of *SIT4* increases the activation of Snf1p, which in turn decreases Ypk1p-Orm signalling to inhibit the *de novo* biosynthetic pathway by possibly reducing SPT activity. As a result, the activation of Sit4p is expected to activate the TORC2-Ypk1p-Orm signalling to stimulate ceramide synthesis through the biosynthetic pathway. On the other hand, Shimobayashi *et al.* showed that TORC1 inhibition activates the kinase Npr1p (by promoting the activation of Sit4p) that directly phosphorylates and activates the Orm proteins. Npr1p-phosphorylated Orm1p and Orm2p stimulate *de novo* synthesis of complex sphingolipids downstream of SPT. As a result, the activation of the TORC2-Ypk1p-Orm mediated ceramide biosynthesis pathway by Sit4p may be coupled to the stimulation of complex sphingolipids when TORC1 activity is inhibited (which relieves the inhibition of Sit4p) to maintain sphingolipid homeostasis. Further studies are required to test this hypothesis. The interplay between ceramide and nutrient signalling is shown in Figure 5.1.

In conclusion, this thesis provides new insights on the molecular mechanisms by which cellular signalling pathways are activated in response to alterations in sphingolipid metabolism and the impact on crucial cellular functions and metabolism (Figure 5.1). This clarification is pivotal to understand sphingolipid functions in more detail and define new strategies to improve human health and extend lifespan.

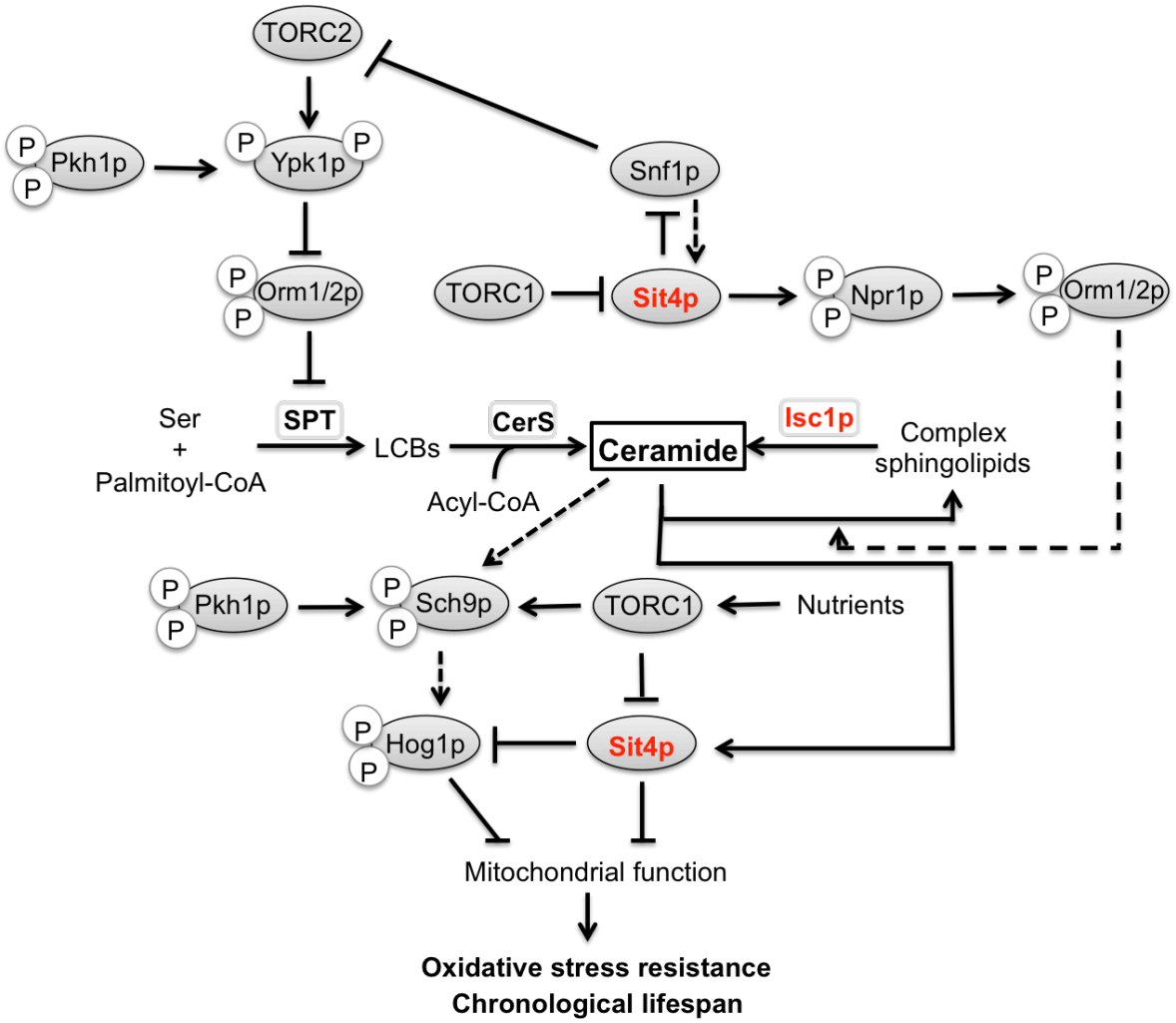

Figure 5.1. Cross talk between ceramide and signalling transduction in the regulation of cell metabolism and longevity. In response to Isc1p-driven ceramide signalling and TORC1-mediated nutrient sensing, the ceramide activated protein phosphatase Sit4p and the AGC protein kinase Sch9p integrate these signals to regulate mitochondrial function, oxidative stress resistance and chronological lifespan in yeast. Additionally, Sit4p emerges as a key regulator of sphingolipid metabolism by integrating TORC1- and TORC2 signalling to regulate complex sphingolipid turnover (TORC1-Sit4p-Npr1p-Orm signalling pathway) and the *de novo* biosynthetic pathway of sphingolipids (TORC2-Pkh1p-Ypk1p-Orm signalling pathway in a Snf1p-dependent manner).

CHAPTER VI

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6.1 References

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Enquanto existir vida em mim, não deixarei de te procurar. Anseio por aquele abraço único e intransmissível. Porque cada abraço é anatomicamente distinto; aquele instante em que os braços anseiam por aquela fusão de corpos e almas é invariavelmente singular. Aquele abraço é como uma partitura musical que só aqueles dois corpos sabem tocar. Adiante, esses corpos comprimem-se num encaixe apertado e característico de uma fusão perfeita de curvas e concavidades. E nesse instante, transportamo-nos no tempo e passamos a viver numa mesma dimensão, onde os desejos se fundem numa dança em ritmo próprio, sublime. Num abraço, naquele momento alígero, roubamos à realidade a distância da saudade, seguramos o tempo num fio invisível, um fragmento do tempo resumido na eternidade de um abraço.

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